To identify a site within the insulin receptor ectodomain which forms a binding pocket for B25 Phe and is responsible for initiating conformational changes required for high affinity binding of insulin we have used a novel photoactive insulin, despentapeptide-(B26-B30) [B25 p-azidophenylalanine-a-carboxamide] insulin (APC insulin). This derivative has a highly photoactive azido group incorporated into the aromatic ring of the B25 phenylalanine amide. APC insulin bound to human insulin receptors overexpressed on a transfected Chinese hamster ovary cell line (P3-A) with an apparent potency of 9-fold relative to that of native insulin and stimulated lipogenesis in rat adipocytes with an average potency equal to porcine insulin. Addition of biotin to the B1 Phe amino group to form despentapeptide-(B26-B30) [B1 (β-biotinylandiocaproyl)phenylalanine B25 p-azidophenylalanine-a-carboxamide] insulin derivative (Bio-APC insulin) did not adversely affect receptor-binding affinity and provided a convenient ligand for purification of cross-linked complexes. The efficiency of receptor cross-linking with these reagents was high (70%). To identify the site(s) of cross-linking, the insulin receptor in P3-A cells was first metabolically labeled with various individual 3H-labeled amino acids and then photolabeled with 125I-Bio-APC insulin, isolated, and digested with Lys-C endoproteinase. The resulting cross-linked peptide fragments were separated by streptavidin-affinity chromatography and sequenced. The smallest identified fragment comprised residues 704-718 of the COOH-terminus of the α-subunit of the insulin receptor. This B25 Phe cross-linked region of the α-subunit lies just upstream of the Exon 11-encoded 12-amino acid COOH-terminal region. Aromatic residues in this predicted α-helical region may form a binding pocket for B25 Phe to initiate conformational changes required for stabilizing the high affinity binding state.

The control of the metabolic and mitogenic functions of insulin is regulated by specific interactions between the hormone and its cell surface receptor. The insulin receptor is a cell membrane spanning glycoprotein tetramer consisting of two α- and two β-subunits. The extracellular domain of the receptor is formed by the ligand binding α-subunit in disulfide linkage to the first 194 amino acids of the β-subunit. The intracellular portion consists of the COOH-terminal 403 amino acids of the β-subunit which includes the tyrosine kinase domain.

In spite of its importance for understanding the mechanism of receptor activation, the location of the ligand-binding region of the insulin receptor remains an unsettled issue. Given the high affinity of insulin for its plasma membrane receptor, it is obvious that multiple interactions between ligand and receptor are required to generate the necessary free energy for specific binding. Among putative residues of insulin involved in receptor binding are the highly conserved sequences A1 to A3 and B23 to B26, among others (1). Replacement of the invariant B25 Phe by 150 degrees in molecule 2. In contrast, most of the COOH-terminal region of the α-subunit lies just upstream of the Exon 11-encoded 12-amino acid COOH-terminal region. Aromatic residues in this predicted α-helical region may form a binding pocket for B25 Phe to initiate conformational changes required for stabilizing the high affinity binding state.

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This paper is dedicated to Dr. Howard Tager, whose elegant studies on mutant insulins and insulin structure-function relationships inspired this work. Dr. Tager died on September 1, 1994.

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more carboxyl-terminal domain that possibly includes sequences encoded by exons 6 and 7 (12–14). To explore further the organization of the insulin-binding domain of the receptor with respect specifically to the binding pocket for B25 Phe, we have used a Chinese hamster ovary cell line which overexpresses the human insulin receptor at very high levels and B25 azidophenylalanyl photoactive insulin derivatives that quantitatively cross-link, radiolabel, and activate the insulin receptor.

EXPERIMENTAL PROCEDURES

Materials—Bovine insulin was obtained from Sigma. 125I-Insulin (porcine) was from Du Pont NEN. [3H]Phe, [3H]Val, [3H]Gly, [3H]Leu, [3H]Arg, and [3H]Tyr were from Amersham Corp. (United Kingdom). Monoclonal antibody (MabIR) was a gift from Dr. Kenneth Stiddle (University of Cambridge, United Kingdom). Phenylmethylsulfonyl fluoride, leupeptin, and pepstatin A were from Boehringer Mannheim (Meylan, France). Tricine was from Bio-Rad. 30% Acrylamide solution was from National Diagnostics (Atlanta, GA). Lys-C endoproteinase was sequencing grade from Boehringer. Polyvinylidene difluoride membrane (Immobilon P) was from Millipore Corp. (Bedford, MA). Nylon membrane was from Amersham (Hybond N). Protein A-agarose was from Pierce. All other reagents were of the highest grade commercially available.

Synthesis of Despentapeptide-(B26-B30) [B25 p-Azidophenylalanylcarboxamidem] Human Insulin (APC Human Insulin)—This compound was prepared by a semisynthetic procedure involving (i) the preparation of despentapeptide-(B26-B30) porcine (human) insulin with all functional groups unprotected (DOP1); (ii) the synthesis (by solution methods) of the tripeptide amide derivative H-Gly-Phe-(p-NH-Boc)-NH2 corresponding to the sequence 23–25 of the insulin B-chain; (iii) the trypsin-catalyzed coupling of the tripeptide amide with DOPI to give despentapeptide-(B26-B30) [B25 Phe-(p-NH-Boc)-α-carboxamidem insulin, and (iv) the removal of the Boc group from the latter compound, and the conversion of the resulting p-NH2 derivative to the final product, APC insulin.

Desocctapeptide-(B26-B30) Porcine (Human) Insulin (DOP1)—This compound was prepared as described previously (5) from porcine insulin, which differs only at B30 from human insulin. H-Gly-Phe-(p-NH-Boc)-NH2—it was purified from its N-benzylcarboxyl derivative by transfer hydrogenation over 10% palladium charcoal catalyst using formic acid as the hydrogen donor. The N-benzoxycarbonyl tripeptide amide (Z-tripeptide amide) was prepared stepwise, starting with H-Phe-(p-NH-Boc)-OH (16) as follows: to a suspension of H-Phe-(p-NH-Boc)-OH (2.8 g) in DMF (30 ml), diisopropylamine (0.3 ml; pH 7.5) and DCC (2.0 g) were added. After 12 h the mixture was diluted with 0.2 M NaHCO3 solution and extracted with ether. The aqueous layer, cooled to 2 °C, was acidified to pH 2.0 in HCl and the oily product extracted into ethyl acetate. The organic layer was washed with water, dried, and concentrated under reduced pressure to dryness. To a solution of the hydrochloride in acetonitrile (60 ml), N-hydroxy-5-norbornene-2,3-dicarboximide (Che-malog) (1.8 g) and N,N'-dicyclohexylcarbodiimide (2.0 g) were added. After 12 h the urea by-product was filtered off and the solvent removed. The residue was dissolved in chloroform (100 ml) and ammonium gas was passed through the solution for 3 h. The precipitated 5-N,N'-dicyclohexylcarbodiimide-Insulin (Bio-DOPI)-NH3 was collected, recrystallized from 95% ethanol (weight, 2.5 g; m.p. > 230 °C) and used in the following synthesis of the dipeptide derivative. To a solution of Z-Phe-(p-NH-Boc)-NH2 (2.5 g) in a mixture of methanol (150 ml) and DMF (20 ml), a 10% palladium charcoal catalyst (1 g) was added followed by formic acid (1 ml). After 1.5 h the catalyst was filtered off, the filtrate concentrated to a small volume and diluted with 1 M cold KHCO3 and the precipitated picrate of the azido insulin analogue was isolated by centrifugation and converted to the hydrochloride as described previously (19). HPLC, as described above, led to the isolation of the insulin analogue in a highly purified form; weight, 6 mg. Upon HPLC rechromatography this product exhibited a single sharp peak with a retention time of 44.9 min. Amino acid analysis after acid hydrolysis was in agreement with the theoretically expected values (data not shown).

Synthesis of Despentapeptide-(B26-B30) [B1 (6-Biotinylamidocaproyl)phenylalanine, B25 p-Azidophenylalanyl-α-carboxamidem Human Insulin (Bio-APC Human Insulin)—This analogue was prepared by a semisynthetic route involving (i) the preparation of [B1 (6-biotinylamidocaproyl)phenylalanine] porcine insulin; (ii) the trypsin-catalyzed condensation of Bio-DOPI with H-Gly-Phe-(p-NH-Boc)-NH2 to give despentapeptide-(B26-B30) [B1 (6-biotinylamidocaproyl)phenylalanine] insulin (Bio-DOP1); and (iii) the trypsin-catalyzed condensation of Bio-DOP1 with H-Gly-Phe-(p-NH-Boc)-NH2 to give desoctapeptide-(B26-B30) [B1 (6-biotinylamidocaproyl)phenylalanine]-Insulin (Bio-APC Human Insulin).
Insulin Receptor-binding Site

[BJ (6-Biotinylamidecaproyl[Phenylalanine] Porcine Insulin—This compound was prepared essentially according to the method of Hofmann et al. (20). Briefly, N^2-[Boc](6-Biotinylamidecaproyl)-bis-(butoxycarbonyl) porcine insulin (21) (62 mg), 6-biotinylamidecaproate N-hydroxysuccinimide ester (BisGMA) (80 mg), and imidazole (28 mg) were dissolved in dimethyl sulfoxide (DMSO) (0.5 ml) and allowed to react for 1 hour at room temperature. Another 1 nmol of chloramine-T was added and the reaction was terminated with 0.1 M acetylthio-}

Preparation of [125I]-APC Insulin—Radioiodination of APC insulin was conducted in subdued light using a described method (25). One mg of APC insulin was dissolved in 0.002 N HCl and 1.6 nmol of APC insulin was incubated in 0.2 n NaHPO_4 (pH 7.0), combined with 1 mCi of Na^125I for 2 min, then added with 1 nmol of chloramine-T. The mixture was thoroughly mixed. 1 nmol of chloramine-T was added and the reaction was terminated with 0.1 M acetylthio-

Phoatoflaijity of the Insulin Receptor—P3-A cells (10^6 cells) were incubated with [125I]-APC insulin (5 x 10^6 cpm) in 3 ml of buffer A with 1% bovine serum albumin overnight in the dark at 4 °C. In a typical experiment, the medium was removed and P3-A cells in 10-cm culture dishes were irradiated at 4 °C for a distance of 2 cm from the light source (Mineralight, model UVG-54, UVP Inc.) equipped with a 284-nm lamp for the indicated time. Insulin receptors with covalently bound APC insulin were immunoprecipitated and analyzed by SDS-

ristamycin (P3-A cells) were plated at density indicated to dwell in 12- or 24-well dishes. After 24 h of incubation, cells were washed twice with Dulbecco’s modified Eagle’s medium deficient in the amino acid with which the receptor was to be labeled. The cells were incubated with 10 ml of Dulbecco’s modified Eagle’s medium without the amino acid for 2 h at 37 °C. The cells were then incubated with 300–500 μCi/dish of [35S]-methionine for 16 h in deficient Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed fetal bovine serum. After incubation, cells were washed with ice-cold buffer A and then incubated with [125I]-Bio-APC insulin (3.5 x 10^6 cpm) in 3 ml of buffer A with 1% bovine serum albumin for 16 h in the dark at 4 °C. After the second incubation, the medium was removed and irradiation was carried out at 4 °C for 30 s in a Petri dish. To obtain sufficient amounts of insulin receptors that are photoaffinity-labeled with Bio-APC insulin, the cells were further incubated in buffer A with Bio-APC insulin (10^7 cpm) for 2 h in the dark at 15 °C. After the third incubation, the medium was removed and irradiation with UV light was carried out under the same conditions as described previously. The cells were then washed with ice-cold buffer A and solubilized in 1 ml of 50 m-Hepe (pH 7.8), 150 m-NaCl, 1% Triton X-100 containing 1 m-methylphenylsulfonyl fluoride, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin for 30 min at 4 °C. After removal of insoluble material by centrifugation, the supernatant was diluted 2:1 with buffer B (20 ml Hepes, 150 mm NaCl, 1% glycercol, 0.1% Triton X-100, pH 7.5) and incubated with 5 μl of anti-human insulin receptor monoclonal antibody (MahI) 83-14 and 50 μl of protein A-agarose. After overnight incubation at 4 °C, the immunoprecipitates were sedimented and washed three times with buffer B.

Protein Transferr—a-Peptide transfer from polyacrylamide gels to membranes (Immel-Now; Millipore, Hybond-N; Amersham) was performed in 26 mm Tris base, 192 m-glycine, 200 NaCl and pH 8.0. Digestion was then carried out with 20 μg/ml Lys-C endoproteinase for the periods indicated at 37 °C in water bath incubator. The reaction was stopped by the addition of 0.15 m-TLCK and the digested fragments were eluted with 100 μl buffer B until the radioactivity of eluate was close to background values.

Streptavidin Complexing—900 μl of eluate containing the digest was incubated with 20 μl of streptavidin-agarose (Pierce) for 16 h. After washing of the agarose four times with 1 ml of buffer B, the agarose was heated for 5 min at 56 °C in 40 μl of 1.5% Laemmli’s sample buffer with or without 100 μm dithiothreitol just prior to PAGE analysis with glycine (26) or Tricine (27) buffers.

Cell Line and Culture—The stably transfected Chinese hamster ovary cell line (P3-A) expresses human insulin receptors with a point mutation in the tetrabasic proreceptor cleavage site (Ly~VA~ -Ala) which does not affect its processing or binding characteristics, as described elsewhere (23). For APC insulin the potency was equal to that of natural porcine insulin (data not shown). The Bio-APC insulin displayed a potency 85% relative to porcine insulin (data not shown).

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Biochemical Evaluation—The ability of the two photoreactive insulin analogues to stimulate, relative to porcine insulin, the conversion of (3-3H)glucose into an organic extractable form by isolated rat adipocytes (lipo genesis) was determined as described previously (22). For APC insulin the potency was equal to that of natural porcine insulin (data not shown). The Bio-APC insulin displayed a potency 85% relative to porcine insulin (data not shown).

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Insulin Receptor-binding Site

Fig. 1. Structure of APC (I) and Bio-APC (II) insulin. These derivatives of insulin contain the following features: residues B26-B30 are deleted and B25 is carboxamidated, a photoreactive group (N₃) occupies the para position of the aromatic ring of B25 Phe. In Bio-APC insulin a 6-biotinamidocaproyl residue has been added for avidin complexing at position B1.

RESULTS

Both APC insulin and Bio-APC insulin (Fig. 1) competed efficiently with [¹²⁵I]iodo-insulin for binding to wheat germ agglutinin-purified insulin receptor preparations (Fig. 2). The results show that the ED₅₀ values, determined as the concentrations of analogues causing half-maximal inhibition of [¹²⁵I]insulin binding, were 1.8 × 10⁻¹⁰ M for APC insulin and 4.8 × 10⁻¹¹ M for Bio-APC insulin, as compared to 1.7 × 10⁻⁹ M for native bovine insulin. Therefore, the relative receptor binding potency for APC insulin was 9.4 and for Bio-APC insulin was 34.9 times higher than that of native insulin. Furthermore, in the stimulation of lipogenesis in rat adipocytes, both derivatives showed potencies similar to porcine insulin.

Insulin binding on intact P3-A cells is shown in Fig. 3. In the experiments performed on whole cells, biphasic displacement curves typical of insulin binding to placental insulin receptor preparations were obtained. P3-A cells bound insulin with high affinity and the ED₅₀ was 1.6 × 10⁻⁹ M. On transformation by the method of Scatchard (28), the P3-A cell line yielded a curvilinear plot, with kₐ = 1.1 × 10⁻⁹ M and k₇ = 2.9 × 10⁻⁸ M. The reason for the discrepancies between the values obtained in the present study and the original values (linear Scatchard plot with kₐ = 5 × 10⁻⁹ M) (23) reported are not known. However, it is possible that a 2-3-fold decrease in total binding sites on the cell surface (6.2 × 10⁶/cell versus 2-3 × 10⁷/cell), possibly an effect of prolonged passage, may account for the more typical curvilinear Scatchard plot obtained in this experiment.

Since aryl azido compounds show higher labeling efficiencies on irradiation at 254 nm than light of longer wavelengths (29), photoaffinity labeling in these experiments was performed at...
cells in 10-cm culture dishes, incubated together at 4 °C for 16 h, were photolyzed for the indicated times as described under "Experimental Procedures." The cells were solubilized and insulin receptors with covalently bound 125I-APC insulin were immunoprecipitated and analyzed by SDS-PAGE.

254 nm. We examined the effect of this short wavelength UV light on both insulin binding and 125I-insulin. Using solubilized wheat germ agglutinin-purified insulin receptors from P3-A cells, insulin binding activity decreased progressively to about 11% of control values over a 30-min UV exposure with a half-maximal effect after 10 min (data not shown). UV exposure of 125I-insulin tracer lead to slightly increased trichloroacetic acid nonprecipitable radioactivity after only 30 s exposure and reduced trichloroacetic acid precipitable radioactivity to 46% of control at 30 min (data not shown). This deiodinating effect of UV light was most rapid during the initial 5 min of exposure and was also half-maximal at 10 min. These results suggested that the optimal conditions of photolysis for avoiding the destructive effects of this shortwave UV light would be exposures of less than 60 s.

The time course of photolytic cross-linking between 125I-APC insulin and insulin receptors on intact P3-A cells is illustrated in Fig. 4. Under these conditions the reaction was essentially complete within 20 s. A single cross-linked species was observed with an apparent molecular mass of 135 kDa on SDS-PAGE. On quantitative analysis the efficiency of cross-linking of 125I-APC insulin to the insulin receptor was roughly 70% (calculated as the incorporation of radioactivity into insulin receptor α-subunit/total radioactivity applied onto the SDS-PAGE).

To determine the site of cross-linking we needed to be able to detect the radioactivity of 125I-photoreactive insulin even after photolysis. We therefore chose Lys-C endoproteinase for digestions since there are no Lys residues in the insulin derivatives we used. The specificity of this proteinase is reported to be high (30). Digestion of APC insulin-insulin receptor complexes with Lys-C endoproteinase gave the results shown in Fig. 5 upon analysis by SDS-PAGE (8 and 15% gels) in the presence or absence of reducing agent.

SDS-PAGE of the cleavage products under nonreducing conditions indicated that a major fragment of 30 kDa is formed along with intermediates of ~114, ~90, ~70, ~42, and 35 kDa (Fig. 5a, DTT(-), lane 2 h). In addition, smaller fragments of ~22 and ~7 kDa were visible after 2 h of digestion (Fig. 5b, DTT(-), lane 2 h). After 18 h of digestion the radioactivity of the 36-kDa fragment decreased and the ~7- and ~4-kDa bands were prominent (Fig. 5b, DTT(+), lane 18 h).

Under reducing conditions a major band appeared at 20 kDa after 2 h of digestion. In addition, ~90-, ~32-, and ~28-kDa bands were visible (Fig. 5a, DTT(+), lane 2 h). After 18 h of digestion the 20-kDa band diminished and the signal intensity of the 5.5- and 3-kDa bands increased. After 24 h of digestion the 5.5- and 3-kDa fragment bands were the most prominent products (Fig. 5b, DTT(+), lane 24 h).

To determine if the 5.5-kDa band contained a part of the insulin receptor, its relative position on the SDS-PAGE was compared with those arising from the photoprobe alone. In the composition of the reaction mixture Triton X-100 did not affect photolysis and migration of the bands in Tricine-SDS-PAGE. 125I-Bio-APC insulin migrated at 4 kDa under nonreducing conditions and at 3 kDa under reducing conditions, representing the insulin A- and B-chains comigrating (Fig. 6, lanes 2–5). After photolysis, 125I-Bio-APC insulin still migrated at ~4 kDa under nonreducing conditions (Fig. 6, lanes 6 and 7). However, an extra band at 4 kDa, in addition to the 3-kDa bands, was observed under reducing conditions, suggesting the formation of a B-chain dimer by photolysis. The 5.5-kDa band after digestion of 125I-Bio-APC insulin and insulin-receptor complex with Lys-C endoproteinase closely differed in size from those bands formed only from 125I-Bio-APC insulin. These findings suggested that the 5.5-kDa band could contain a peptide derived from the insulin receptor, and we therefore examined the amino acid sequence of this 5.5-kDa fragment.

In order to obtain sufficient material for microsequencing, P3-A cells in five 10-cm culture dishes (1–2 × 10^6 cells/dish) were metabolically labeled with various individual 3H-labeled amino acids and successively photoaffinity labeled with 125I-Bio-APC insulin (see "Experimental Procedure"). The photoaffinity labeled receptor was digested with Lys-C endoproteinase for 24 h. The resulting photoaffinity labeled fragments were isolated by incubation with streptavidin-agarose and then analyzed on SDS-PAGE. The protein bands in the SDS gels were transferred to polyvinylidene difluoride or nylon membranes. After autoradiography, the radioactive 5.5-kDa bands were cut out and eluted with 88% formic acid. The eluate was directly applied to an amino acid sequencer. In a typical experiment 40% of the total counts (usually 5–6 million counts of 125I-Bio-APC insulin) was bound to the receptor. 80% of the 125I-Bio-APC insulin and insulin-receptor complex was isolated after immunoprecipitation with MabIR 83–14. After digestion, 80% of radioactivity became attached to streptavidin-agarose; ~10% of total radioactivity initially applied to the SDS gel was found in 5.5-kDa fragment. Protein transfer yielded about 50% recovery and typically 20,000–40,000 cpm of γ radioactivity was subjected to an amino acid sequence analysis. In experiments using [3H]Phe or [3H]Val for labeling the insulin receptors, the radioactive residues found on amino acid sequence analysis of the 5.5-kDa fragment obtained under reducing conditions corresponded uniquely with the sequence of peptide Thr^794 to Lys^718, as predicted by a computer analysis of potential receptor peptides generated by Lys-C endoproteinase digestion (Fig. 7). The extra peaks at positions 14 and 19 were seen even under reducing conditions and represented [125I]iodobenzene at A14 and A19 (tyrosines) in the probe; as shown in Fig. 8 (DTT(+)) Tricine-SDS-PAGE gave a broad smear of radioactivity. In separate experiments this was found to be due to a mixture of reduced insulin A- and B-chains (data not shown). Accordingly, the observed 125I radioactivity at these positions probably originated from small amounts of co-migrating insulin A-chain, but not from the B-chain since the NH2 terminus of the B-chain was blocked by bovine insulin. In labeling experiments using [3H]Leu or [3H]Tyr, amino acid sequence analysis of the ~7-kDa fragment obtained under nonreducing conditions (Fig. 8, DTT(−)) also yielded radioactive tritium peaks at appropriate positions corresponding to the sequence of peptide Thr^794-Lys^718 (Fig. 7). To determine whether this fragment extended to the most COOH-terminal residue of the α-subunit (Ser^731), [3H]Gly- or [3H]Arg-labeled insulin receptors were also digested and analyzed. However, no [3H]radioactive peaks corresponding to these amino acids were observed.
Our results demonstrate that active insulin derivatives which possess a highly photoactive group in the para position of the aromatic ring of B25 phenylalanine become cross-linked to the COOH-terminal domain of the B-chain for receptor interaction. Re- placement of B25 phenylalanine by serine, leucine, or homophenylalanine leads to a 100-fold decrease in receptor affinity (5), but this decrease in affinity is partially reversed (by up to 40-fold) when COOH-terminal residues B26 to B30 are deleted. These findings indicate the importance of aromaticity at B25 and support the existence of a specific aromatic binding pocket for this residue in the a-subunit. In our design of a receptor probe we speculated that a possible decrease in affinity due to the modification of B25 Phe with the azido group would be reversed by deletion of the COOH-terminal 5 residues and amida- tion of B25. Tager had earlier shown that such a derivative having B25 tyrosine amide had 3-fold increased receptor affinity (5). The high affinity binding of APC insulin achieved in our experiments is consistent with his findings.

Although we have not identified the specific amino acid residue(s) in this peptide which is covalently linked to the B25 Phe, it is in a relatively hydrophobic region that is predicted to form an a-helix. From the amino acid sequence of the insulin receptor Phe705 and Tyr706 together with the adjacent Phe701 could form an aromatic cluster in an a-helical configuration. These findings, however, are in contrast to previous suggestions based on molecular graphics simulations that Phe89 of the insulin receptor interacts with Phe25 of the insulin molecule (7). The earlier conclusions were based on the modeling and observations of decreased insulin binding in studies of mutated receptors with substitutions for Phe residues 88 or 89. However, derivatives used in most previous receptor cross-linking studies, which have utilized photoactivatable groups attached via linkers to residues of insulin that are not directly involved in insulin binding, the APC insulins incorporate a relatively small azido group on the para position of the phenylalanyl side chain of B25, a residue predicted to participate directly in receptor interaction of B25 phenylalanine by serine, leucine, or homophenylalanine leads to a 100-fold decrease in receptor affinity (5), but this decrease in affinity is partially reversed (by up to 40-fold) when COOH-terminal residues B26 to B30 are deleted. These findings indicate the importance of aromaticity at B25 and support the existence of a specific aromatic binding pocket for this residue in the a-subunit. In our design of a receptor probe we speculated that a possible decrease in affinity due to the modification of B25 Phe with the azido group would be reversed by deletion of the COOH-terminal 5 residues and amidation of B25. Tager had earlier shown that such a derivative having B25 tyrosine amide had 3-fold increased receptor affinity (5). The high affinity binding of APC insulin achieved in our experiments is consistent with his findings.
Insulin Receptor-binding Site

Amino acid sequence analysis of 5.5- or 7-kDa insulin receptor fragments. Metabolic labeling of receptors was carried out using tritiated Phe, Val, Gly, or Arg. After isolation and proteolytic digestion for 24 h, 5.5-kDa fragments were obtained from SDS-PAGE under reducing conditions and sequenced. In separate labeling experiments with tritiated aromatic substrates, 7-kDa fragments were obtained under nonreducing conditions and analyzed. See "Experimental Procedures" for details. Note that the more prominent peaks in reduced samples, due to A-chain tyrosines (residue numbers 14 and 19), arose from smearing of the reduced insulin chains in the Tricine-SDS-PAGE system; see Fig. 8 and text for details.

Fig. 7. Amino acid sequence analysis of 5.5- or 7-kDa insulin receptor fragments. Metabolic labeling of receptors was carried out using tritiated Phe, Val, Gly, or Arg. After isolation and proteolytic digestion for 24 h, 5.5-kDa fragments were obtained from SDS-PAGE under reducing conditions and sequenced. In separate labeling experiments with tritiated aromatic substrates, 7-kDa fragments were obtained under nonreducing conditions and analyzed. See "Experimental Procedures" for details. Note that the more prominent peaks in reduced samples, due to A-chain tyrosines (residue numbers 14 and 19), arose from smearing of the reduced insulin chains in the Tricine-SDS-PAGE system; see Fig. 8 and text for details.

direct evidence that B25 contacts Phe was not obtained. On the other hand, their assumptions regarding the importance of aromatic-aromatic interactions in B25 Phe binding lend support to our findings. Such interactions are common in globular proteins and probably are very important for stabilization of protein tertiary structure (35, 36). Furthermore, aromatic side chains often form hydrophobic pockets that preferentially bind aromatic substrates (37). Accordingly, the aromatic residues in the COOH-terminal region of the α-subunit may form a binding pocket necessary for high affinity binding of insulin.

B25 Phe has long been recognized an important residue in insulin binding. Aromaticity at this point is also conserved in the insulin-like growth factors. Residues 23-26 of the COOH-terminal region of the B-chain are considered to be part of the active site of the hormone. A point mutation at B25 resulting in a leucine for phenylalanine substitution has been found in a diabetic patient (4) and is associated with greatly reduced binding affinity. This region also plays a crucial role in dimer formation within the zinc-insulin hexamer. In a predicted model for insulin and insulin-receptor interaction the β-aromatic ring of B25 Phe participates in an active way in initiating conformational changes that occur in the insulin-receptor complex (5). Thus the filling of a receptor binding pocket by the β-aromatic side chain of B25 may well facilitate or initiate additional conformational changes that probably occur both in the COOH-terminal B-chain domain of insulin and in the receptor site (5, 38). Since the insulin receptor was photoaffinity labeled at a saturating concentration of Bio-APC insulin in order to determine the amino acid sequence at the cross-linked site(s), there is a possibility that the binding site we have detected might be a low affinity site. However, Shoelson et al. (38) reported that each holoreceptor (ααββ) was cross-linked by one molecule of insulin even at saturating concentrations (10^-6 M) using a somewhat similar photoreactive insulin analogue (Bpa insulin) which binds to the receptor with high affinity, and that this is sufficient to activate a reaction cascade starting with autophosphorylation. The cross-linking site in the receptor for this insulin derivative has not yet been reported. It has also been observed that each receptor half-molecule (αβ) binds insulin with low affinity (39). These findings and other data suggest the possibility that to achieve the high affinity binding state one insulin molecule must interact with sites in both α-subunits within each holo-receptor (40). After a high affinity binding state has been achieved, a lower affinity site might be created to bind a second insulin molecule. Insulin binding to the lower affinity site may occur in a different orientation, however, since the previously mentioned Bpa insulin, which has a photoreactive benzoylphenylalanine residue at B25, only detected a single high affinity site (38). We thus believe that the site we have identified is involved in the formation of the high affinity binding state.

The insulin binding region identified in this study is also of interest because this occurs just adjacent to the sequence encoded by exon 11. Alternative splicing of a single gene tran-
script yields two insulin receptor mRNA species and two receptor isoforms, HIR-A and HIR-B, which differ by the 12-amino acid sequence encoded by exon 11. This additional sequence is inserted at residue 716 of HIR-A (exon 11+) to yield HIR-B (exon 11+) (41, 42, 43). The two isoforms possess distinct functional properties and are expressed in a tissue-specific fashion (44–48). In addition to the pathophysiological significance of changes in HIR A/B expression in the skeletal muscle associated with development of non-insulin-dependent diabetes mellitus (49–51), we have postulated and demonstrated that the absence of this 12-amino acid segment encoded by exon 11 affects the folding and/or conformation of the proreceptor so as to confer decreased sensitivity to insulin.

Thus cleavage at the α,β-subunit junction (residues 720–723) has been proposed as candidates for binding regions by several groups (6–11). A more COOH-terminal domain possibly within this COOH-terminal region of the α-subunit. On the other hand, other regions are also potentially important for high affinity binding, our data also are compatible with the results obtained in studies using anti-insulin receptor antibodies that have both been proposed as candidates for binding regions by several groups (6–11).

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