# Identification and Characterization of the Ligand-Binding Domain of Insulin Receptor by Use of an Anti-Peptide Antiserum against Amino Acid Sequence 241–251 of the α Subunit†

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ABSTRACT: We previously reported that a 23-kDa receptor proteolytic fragment containing an insulin-binding site was localized within residues 205–316 in the cysteine-rich region of the insulin receptor α subunit and postulated that sequence 241–251 plays a major role in insulin binding [Yip, C. C., Hsu, H., Patel, R. G., Hawley, D. M., Maddux, B. A., & Goldfine, I. D. (1988) *Biochem. Biophys. Res. Commun. 157*, 321–329]. In the present study, we have used an antiserum raised against a synthetic peptide containing sequence 241–251 to test this postulate and to study the role of sequence 241–251 in insulin binding. The antiserum immunoprecipitated the 23-kDa fragment, confirming our sequence assignment of this fragment. It also immunoprecipitated the intact α subunit of the insulin receptor that had been denatured by reduction and alkylation. However, sequence 241–251 in the native receptor was inaccessible to the antiserum since the antiserum did not block [$^{125}$I]iodoinsulin binding and did not precipitate either photoaffinity-labeled insulin receptors or insulin receptors labeled with $^{125}$I. However, using a radioactive photoaffinity probe ([$^{125}$I]-AZAP-insulin) that allows cleavage and removal of insulin after photolabeling, we found that sequence 241–251 became accessible to the antiserum after removal of insulin. We conclude therefore that sequence 241–251 forms part of the insulin-binding domain of the insulin receptor and that the binding of insulin to the receptor induces a conformational change that allows exposure of this domain after removal of insulin. Such a conformational change may play a role in activation of the receptor and transmembrane signaling.

Insulin receptors are disulfide-linked oligotetramers composed of two heterodimers each consisting of a 130-kDa α subunit and a 90-kDa β subunit. The receptor amino acid sequence, as deduced from the cloned cDNA, indicates that the α subunit is totally extracellular and is disulfide-linked to the β subunit which contains a single membrane-spanning domain (Ullrich et al., 1985; Ebina et al., 1985). These structural features have been confirmed experimentally (Grunfeld et al., 1985). Photoaffinity labeling and chemical cross-linking studies have demonstrated that insulin binds primarily to the α subunit (Yip et al., 1980; Pilch & Czech, 1980). Insulin binding to the α subunit leads to the activation of the intracellular tyrosine kinase domain of the β subunit (Kasuga et al., 1982; Petruzelli et al., 1982; Van Obberghen et al., 1983) which involves the binding of ATP and autophosphorylation of the β subunit (Tornqvist et al., 1987; Chou et al., 1987; White et al., 1988). Although it is likely that insulin binding to the receptor α subunit induces conformational changes which then influence the β subunit, very little is known about this process.

On the basis of results obtained from the use of synthetic peptides, proteolytic digestion of photoaffinity-labeled insulin receptor, and deletion mutagenesis of the insulin receptor, we have recently postulated that one site of insulin interaction with the α subunit is located in amino acid sequence 205–316 in the cysteine-rich region of the subunit and that the sequence PPYYHFQDW (residues 243–251) interacts with insulin (Yip et al., 1988). Recently, we have also demonstrated by site-specific mutagenesis that an alteration of this sequence from *PP*YY*H*FQDW to *RR*YY*D*FQDW increased the binding affinity of the receptor and that the sensitivity to insulin was also increased in cells transfected with the mutant receptor (Rafaeloff et al., 1989). These observations strongly suggested that sequence 243–251 in the receptor α subunit plays an important role in the binding of insulin. In order to further study this insulin-binding domain, we have now raised an antiserum against sequence 241–251. Herein we present data that suggest insulin induces a conformational change in this region of the receptor.

## Materials and Methods

*Materials.* The following materials and reagents were obtained from the sources indicated: bovine insulin (Zn crystals), a gift from Connaught-Novo Laboratories Ltd. (Toronto); *N*-[4-[(4′-azido-3′-[$^{125}$I]iodophenyl)azo]benzoyl]-3-aminopropanoate *N*-succinimidyl ester (2200 Ci/mmol, Denny-Jaffe reagent) from Du Pont Canada Inc.; carrier-free [$^{125}$I]iodine (IMS-30) from Amersham Canada Ltd.; *Staphylococcus aureus* V8 endoproteinase Glu-C (EC 3.4.21.19) from Boehringer Mannheim, Canada; dithiothreitol and reagents for SDS–PAGE[1] from Bio-Rad Laboratories (Canada); sodium

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; [$^{125}$I]AZAP-insulin, *N*-[4-[(4′-azido-3′-[$^{125}$I]-iodophenyl)azo]benzoyl](3-aminopropyl)insulin; $B_{29}$-MAB-[$^{125}$I]iodoinsulin, $N^{\alpha B29}$-(monoazidobenzoyl)[$^{125}$I]iodoinsulin; MBS, *m*-maleimidobenzoate *N*-succinimidyl ester.

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dithionite from Fisher Scientific Co.; Sepharose–protein A from Pharmacia (Canada); keyhole limpet hemocyanin from Behring Diagnostics (La Jolla, CA); *m*-maleimidobenzoate *N*-succinimidyl ester from Pierce Chemical Co. (Rockford, IL). Synthetic peptide CPPPYYHFQDW-$NH_2$ (peptide II) corresponding to sequence 241–251 with a purity of better than 95% was prepared by iAF BioChem International, Inc. (Laval, Quebec). The radioactive photoprobes of insulin, $B_{29}$-MAB-[$^{125}$I]iodoinsulin and [$^{125}$I]AZAP-insulin, were prepared as described (Yip et al., 1980; Ng & Yip, 1985). ARS-2, a human autoimmune serum against the insulin receptor, was obtained from a patient in Toronto.

*Production of Anti-Peptide Serum.* Peptide II was conjugated to keyhole limpet hemocyanin with *m*-maleimidobenzoate *N*-succinimidyl ester (MBS) as the coupling reagent (Liu et al., 1979). Rabbits were immunized three times with 0.5 mg of the conjugate in complete Freund's adjuvant at intervals of 10–12 days followed by three times with incomplete adjuvant. Where indicated, immunoglobulin was prepared from the serum by ammonium sulfate precipitation at 50% saturation. The presence of anti-peptide antibodies in the serum was detected by the specific binding of $^{125}$I-peptide II prepared as described previously (Yip et al., 1988). An aliquot of $^{125}$I-peptide II, containing approximately 200 000 cpm of radioactivity, was incubated at 4 °C overnight with 10 μL of preimmune serum or immune serum and 10 μg of the various peptides as indicated in a final volume of 100 μL in 40 mM phosphate buffer, pH 7.4, containing 150 mM NaCl. Each sample was then incubated with 7 mg of Sepharose–protein A beads at 4 °C for 2 h in a rotator to ensure good mixing. The beads were then washed three times with 1 mL of the buffer followed by three washes with 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl and 0.1% Triton X-100. Radioactivity bound to the beads was determined by counting in a γ scintillation counter.

*Preparation of Photoaffinity-Labeled Insulin Receptor and Its α Subunit.* Human placental insulin receptors were solubilized and affinity purified on insulin–Affi-Gel 10 (Fujita-Yamaguchi et al., 1983). The partially purified receptor, approximately 10 μg of protein, was photoaffinity labeled (Yip, 1984) with approximately $10^7$ cpm of either $B_{29}$-MAB-[$^{125}$I]iodoinsulin or [$^{125}$I]AZAP-insulin as described previously (Ng & Yip, 1985). The structural features of the two types of photoreactive insulins are schematically presented in Figure 1A. In [$^{125}$I]AZAP-insulin the radioactive iodine atom is located on the photoreactive moiety of the probe, whereas in $B_{29}$-MAB-[$^{125}$I]iodoinsulin the insulin moiety of the probe is iodinated. Figure 1B illustrates the results of azo cleavage on the two types of photoaffinity-labeled receptors and the production of labeled α subunit by reduction and alkylation. In the case of receptors labeled with the cleavable [$^{125}$I]-AZAP-insulin, azo cleavage releases insulin from the labeled receptor, leaving the radioactive iodophenyl group covalently linked to the receptor. Receptors labeled with the noncleavable $B_{29}$-MAB-[$^{125}$I]iodoinsulin are not affected. Experimentally, azo cleavage was carried out with sodium dithionite (Denny & Blobel, 1984). After treatment with dithionite the reaction mixture was desalted through Sephadex G-75 in 50 mM phosphate buffer, pH 7.4, containing 0.9% NaCl and 0.1% Triton. The labeled receptor was recovered in the void volume. Radioactive photolabeled receptor α subunit was prepared by reduction and alkylation of the photolabeled receptor, followed by SDS–PAGE and elution of the radioactive α subunit band from the SDS gel with 10% methanol as previously described (Yip et al., 1988).

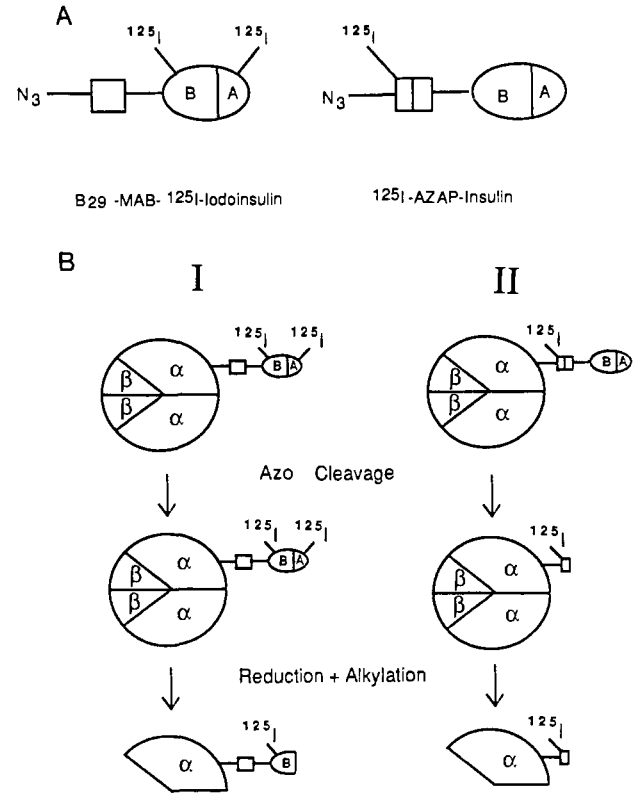


FIGURE 1: (A) Schematic illustration of the structure of $B_{29}$-MAB-[$^{125}$I]iodoinsulin and [$^{125}$I]AZAP-insulin. Insulin is represented by the oval in which A and B denote respectively the A and B chains. The square represents the benzoyl group in MAB-iodoinsulin and the (phenylazo)benzoyl group in AZAP-insulin. The vertical line in the latter square indicates the azo linkage which is cleaved by dithionite. (B) Schematic illustration of the effect of azo cleavage by dithionite and the reduction and alkylation of insulin receptors photoaffinity-labeled with either (I) $B_{29}$-MAB-[$^{125}$I]iodoinsulin or (II) [$^{125}$I]-AZAP-insulin. In (II) insulin is released from the photoaffinity-labeled receptor, and radioactivity is transferred to the receptor subunit, whereas in (I) the B chain of insulin remains cross-linked to the subunit. For clarity, insulin is shown cross-linked to one α subunit in a receptor oligomer.

*Preparation of Proteolytic 23-kDa Fragment of Photolabeled Receptor α Subunit.* The reduced and alkylated labeled α subunit was digested at 37 °C with endoproteinase Glu-C in 50 mM ammonium bicarbonate (Houmard & Drapeau, 1972) as described previously to generate a 23-kDa-labeled fragment (Yip et al., 1988). The endoproteinase Glu-C digest was separated on gradient slab SDS gels of 9–26% (DeWald et al., 1986). The radioactive 23-kDa fragment was extracted from the gel with 10% methanol and lyophilized. The lyophilized samples were reconstituted in 50 mM ammonium bicarbonate, and excess SDS present in the sample was removed by precipitation with an equal volume of 50 mM KCl (Suzuki & Terada, 1988). Before immunoprecipitation, the sample was concentrated and buffer exchanged for phosphate-buffered saline on a Centricon-30 microconcentrator (Amincon).

*Preparation of Insulin Receptor Labeled with $^{125}$I.* Partially purified insulin receptor (100 μL, containing approximately 1 μg of receptor protein) was added to 1 mCi of carrier-free $^{125}$I in 60 μL of 0.4 M phosphate buffer, pH 7.4. Iodination was initiated by addition of 10 μL of chloramine T (1 mg/mL in the phosphate buffer) to the mixture kept in ice. After 6 min, another 10 μL of the oxidant was added. The reaction was terminated after 1 min by the addition of 20 μL of sodium metabisulfite (2 mg/mL in water) and 10 μL of KI (0.1 M). The mixture was transferred to a Pharmacia P-10 prepacked

Table I: Immunoprecipitation of $^{125}$I-Peptide II[a]

| addition | cpm precipitated |
|---|---|
| preimmune serum | 1761 |
| antiserum AP-II | 12488 |
| +peptide II | 3290 |
| +peptide I | 12714 |
| +insulin | 12715 |
| +glucagon | 12797 |

[a] Immunnoprecipitation was carried out as described in the text. Results are presented as average of duplicate determinations. Peptide I was a synthetic peptide (YQDLHHKCKNSR-$NH_2$) containing residues 260–270 of the insulin receptor.

column equilibrated with 50 mM Tris-HCl, pH 7.4, containing 0.1% Triton X-100 and 0.1% bovine albumin. The column was eluted with the same buffer in 1-mL fractions. Fractions containing the iodinated protein, usually fractions 3 and 4, were pooled and kept at 4 °C for use.

*Immunoprecipitation.* Immunoprecipitation was carried out as previously described (Phillips et al., 1986) with an amount of immunoglobulin equal to that present in 10–20 μL of the original serum. Sepharose–protein A was used to recover the immune complex. The complex was solubilized from the Sepharose beads by boiling for 5 min in SDS sample buffer containing 50 mM dithiothreitol. The solubilized material was analyzed by SDS–PAGE in either 7.2% gels or gradient gels of 9–26%. Radioautograms of the dry gels were obtained with Kodak X-Omat AR films and Lightning-Plus enhancing screens. Exposure time ranged from 2 to 7 days.

## Results

*(I) Immunoreactivity of an Antiserum to Peptide II.* Rabbits immunized with peptide II conjugated to keyhole limpet hemocyanin produced antibodies against the synthetic peptide. Table I shows the specific immunoprecipitation of $^{125}$I-labeled peptide II by antiserum AP-II obtained from one of the immunized animals. Immunoprecipitation of $^{125}$I-labeled peptide II was inhibited by unlabeled peptide II but not by either another insulin receptor peptide (peptide I, containing the amino acid sequence 260–270) or insulin and glucagon.

*(II) Effect of Antiserum on Insulin Binding.* We then examined the effect of antiserum AP-II on insulin binding. Figure 2 shows that AP-II did not inhibit the binding of insulin to solubilized and affinity-purified insulin receptor from human placenta, whereas insulin binding was inhibited by ARS-2, a human autoimmune serum against the insulin receptor. Also, AP-II did not inhibit insulin binding by placental membranes and their ability to be labeled by the two photoprobes (data not shown).

*(III) Immunoprecipitation of Denatured Photoaffinity-Labeled Receptors.* As shown in Figure 3A (lane 1), AP-II immunoprecipitated the denatured α subunit obtained by reduction and alkylation of insulin receptor photoaffinity labeled with the cleavable [$^{125}$I]AZAP-insulin. In addition, several minor bands of smaller size, likely degradation products of the α subunit, were also precipitated by AP-II. Immunoprecipitation of the labeled α subunit and the minor bands was abolished by the addition of peptide II (Figure 3A, lane 2). About 40% of the radioactivity added was precipitated by the antiserum (10 μL). In contrast, the precipitation of the labeled subunit by the ARS-2 was insignificant (Figure 3A, lanes 3 and 4). In these experiments the photoaffinity-labeled receptors were treated with dithionite to release the insulin molecule before reduction and alkylation. AP-II also precipitated denatured α subunits prepared by reduction and alkylation of receptors photoaffinity labeled with either the noncleavable $B_{29}$-MAB-[$^{125}$I]iodoinsulin or the cleavable

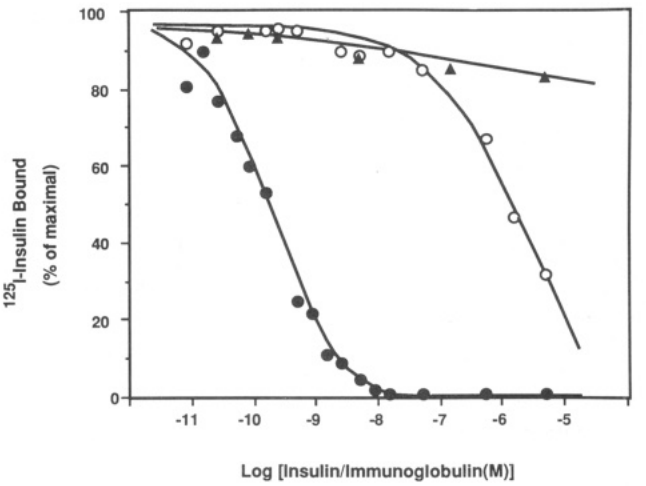


FIGURE 2: Effects of anti-receptor serum ARS-2 and antiserum AP-II on insulin binding. Insulin binding was carried out as described previously (Haynes et al., 1986) with $^{125}$I-Tyr$^{A14}$-insulin and human placental membranes. Incubation was at 4 °C for 20 h. Membrane-bound insulin was separated by precipitation with poly(ethylene glycol) with bovine γ-globulin as carrier protein. Each point represents the average of a duplicate determination. Insulin (●), immunoglobulin from antiserum AP-II (▲), and anti-receptor serum ARS-2 (○) were used at the concentrations indicated for competition.

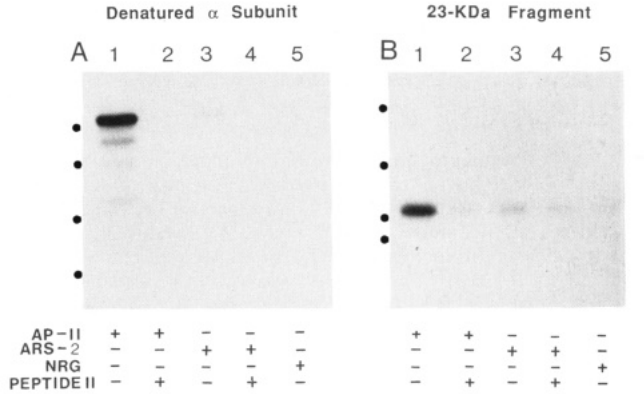


FIGURE 3: (A) Radioautogram showing the precipitation of denatured insulin receptor α subunit by antiserum AP-II. Partially purified human placental insulin receptors were photoaffinity labeled with [$^{125}$I]AZAP-insulin. After cleavage of insulin from the labeled receptor by treatment with dithionite, the labeled α subunit was prepared by reduction and alkylation of the labeled receptor. Immunoprecipitation of the labeled α subunit by immunoglobulins prepared from the antiserum AP-II, an anti-receptor serum ARS-2 and a preimmune serum (NRG) was carried out as described previously (Phillips et al., 1986) and in the text. The immunoprecipitates were separated by SDS–PAGE on 7.2% gel. Positions of standard molecular mass markers of 116, 97, 66, and 43 kDa are indicated in descending order by solid circles. (B) Radioautogram showing immunoprecipitation of the photolabeled 23-kDa receptor fragment. Partially purified human placental insulin receptor was photoaffinity labeled with [$^{125}$I]AZAP-insulin, followed by azo cleavage, reduction, and alkylation to generate the labeled α subunit. The labeled 23-kDa fragment was produced by exhaustive digestion of the subunit with endoproteinase Glu-C as previously described (Yip et al., 1988). Precipitation of the fragment by immunoglobulins from AP-II, an anti-receptor serum ARS-2, and a preimmune serum NRG was carried out as described in the text. The immunoprecipitates were separated by SDS–PAGE on a gradient gel of 9–26%. The solid circles mark the positions of standard molecular mass markers of 43, 31, 22, 14, kDa in descending order.

[$^{125}$I]AZAP-insulin without azo cleavage to release the insulin (data not shown).

We have previously reported that endoproteinase Glu-C digestion of the reduced and alkylated α subunit produced a 23-kDa fragment which was immunoprecipitated by four monoclonal antibodies against the receptor α subunit (Yip et

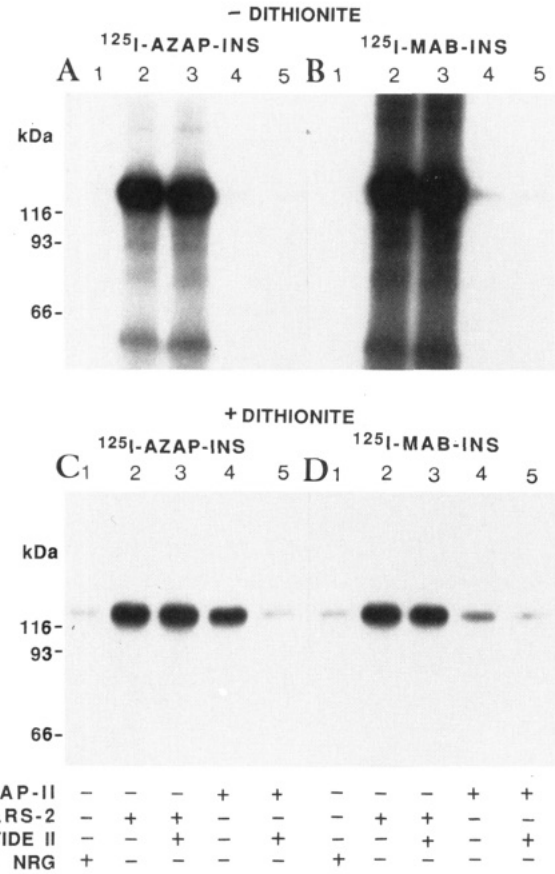


FIGURE 4: Radioautograms showing immunoprecipitation of photoaffinity-labeled insulin receptor. Receptors were photoaffinity labeled with (A) the cleavable [$^{125}$I]AZAP-insulin, (B) the noncleavable $B_{29}$-MAB-[$^{125}$I]iodoinsulin, (C) [$^{125}$I]AZAP-insulin followed by treatment with dithionite to affect azo cleavage to remove insulin, and (D) $B_{29}$-MAB-[$^{125}$I]iodoinsulin followed by treatment with dithionite as in (C). The photoaffinity-labeled receptors were then precipitated with immunoglobulin from the antiserum AP-II, the anti-receptor serum ARS-2, or preimmune rabbit serum NRG in the absence (−) or the presence (+) of peptide II. The immunoprecipitate was solubilized in SDS and 50 mM dithiothreitol before SDS–PAGE in a 7.2% gel. Exposure time was 4 days.

al., 1988). Figure 3B (lane 1) shows that this 23-kDa fragment was precipitated by AP-II and that the precipitation of the 23-kDa fragment was abolished by the addition of peptide II (lane 2). Approximately 50% of the radioactivity added was precipitated by the antiserum (10 μL). ARS-2 did not precipitate the 23-kDa fragment.

*(IV) Immunoprecipitation of Photoaffinity-Labeled Native Undenatured Receptors.* Since AP-II was able to immunoprecipitate the reduced and alkylated receptor α subunit as well as its proteolytic 23-kDa fragment (Figure 3) but did not inhibit insulin binding (Figure 2), we determined whether AP-II could interact with the native receptor. Insulin receptors were first photoaffinity labeled with either $B_{29}$-MAB-[$^{125}$I]-iodoinsulin or [$^{125}$I]AZAP-insulin and then incubated with either AP-II or ARS-2. As expected, receptors photoaffinity labeled with either photoprobe were precipitated by ARS-2 (Figure 4A,B, lanes 2). The precipitation of the labeled receptor by ARS-2 was not diminished by the addition of peptide II (Figure 4A,B, lanes 3), indicating that the immunoprecipitation by this antiserum did not involve the epitope represented by sequence 241–251. In contrast to ARS-2, AP-II did not immunoprecipitate the photoaffinity-labeled receptor preparations (Figure 4A,B, lanes 4).

However, when the receptors were photoaffinity labeled with the cleavable photoprobe, [$^{125}$I]AZAP-insulin, and then treated

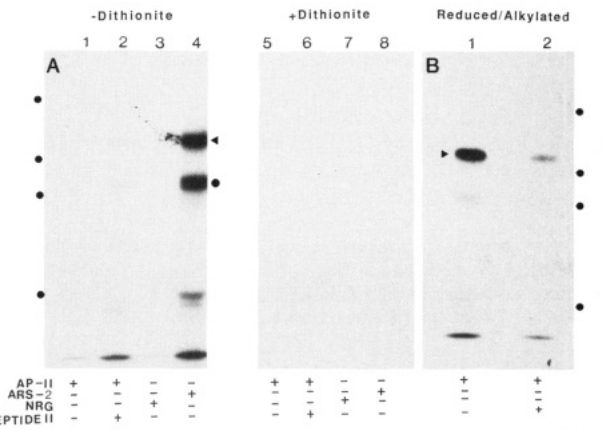


FIGURE 5: Radioautograms showing immunoprecipitation of $^{125}$I-labeled insulin receptor. (A) In lanes 1–4 $^{125}$I-labeled receptors were precipitated with immunoglobulin from the antiserum AP-II, preimmune rabbit serum NRG, or the anti-receptor serum ARS-2. In lanes 5–8 $^{125}$I-labeled receptors were treated with dithionite as described in the text and then precipitated with immunoglobulin from AP-II, NRG, or ARS-2. (B) $^{125}$I-Labeled receptors were reduced and alkylated (Yip et al., 1988) and then immunoprecipitated with immunoglobulin from AP-II in the absence (lane 1) or the presence of peptide II (lane 2). The immunoprecipitates were solubilized in SDS and 50 mM dithiothreitol before SDS–PAGE in 7.2% gel. The radioactive α subunit (▲) and β subunit (●) are indicated. The solid circles mark the positions of standard molecular mass markers of 200, 116, 97, and 43 kDa in descending order.

with dithionite to release insulin through azo cleavage, the labeled receptors became precipitable by AP-II (Figure 4C, lane 4), and the addition of peptide II reduced the immunoprecipitation (Figure 4C, lane 5) to the same level as that precipitated by preimmune immunoglobulin (Figure 4C, lane 1). Further, when receptors labeled with the noncleavable probe, $B_{29}$-MAB-[$^{125}$I]iodoinsulin, were treated with dithionite under the same conditions, only a small amount of the labeled receptor was precipitated by the AP-II (Figure 4D, compare lane 4 with lane 5). The band containing the labeled α subunit in each lane was excised for the determination of radioactivity. After treatment with dithionite, the amount of AZAP-labeled receptor precipitated by AP-II as measured by the radioactivity associated with the band was increased 10-fold whereas there was less than a 2-fold increase in the case of the MAB-labeled receptor. In all cases, dithionite treatment of the labeled receptors reduced their precipitation by the anti-receptor serum ARS-2 by about 90% (Figure 4C,D, lanes 2).

*(V) Immunoprecipitation of $^{125}$I-Labeled Insulin Receptors.* In order to eliminate the possibility that the inability of the AP-II to precipitate the undenatured photoaffinity-labeled receptors was caused by photolabeling, receptors labeled with [$^{125}$I]iodine were tested for their ability to react with AP-II. Figure 5A (lane 4) shows that undenatured $^{125}$I-labeled receptors were precipitated by ARS-2, as evident by the presence of the radioactive α and β subunit bands. Like the photolabeled receptors, the undenatured $^{125}$I-labeled receptors were not precipitated by AP-II (lane 1). We studied also the effect of dithionite treatment of the $^{125}$I-labeled receptors on their reactivity with ARS-2 and AP-II. Figure 5A shows that, after dithionite treatment, the $^{125}$I-labeled receptors remained unreactive with AP-II (lane 5). However, dithionite-treated receptors were no longer precipitated by ARS-2 (lane 8). Dithionite treatment of unlabeled native receptors also resulted in their complete loss of insulin-binding activity, and the treated receptors could no longer be photolabeled (data not shown). When the $^{125}$I-labeled receptors were first incubated with insulin (10 nM) overnight at 4 °C before treatment with dithionite, there was no binding by either ARS-2 or AP-II.

However, after both reduction and alkylation, $^{125}$I-labeled receptors were precipitated by AP-II (Figure 5B as demonstrated by the presence of the labeled $\alpha$ subunit band. This precipitation was blocked by peptide II. The trace amount of $\beta$ subunit detected was likely due to the precipitation of incompletely reduced receptor.

## Discussion

We have previously observed that a labeled 23-kDa fragment of the insulin receptor was obtained by endoproteinase Glu-C digestion of receptors photoaffinity labeled with [$^{125}$I]AZAP-insulin and postulated that this fragment contained an insulin-binding site and spanned residues 205–316 in the cysteine-rich region of the receptor $\alpha$ subunit (Yip et al., 1988). A synthetic peptide, PPYYHFQDW, corresponding to residues 243–251 bound to immobilized insulin, and the binding of this peptide was inhibited by solubilized insulin receptor (Yip et al., 1988). In addition, receptors in which this sequence has been altered to RRYYDFQDW by site-specific mutagenesis showed an increase in the affinity of binding of insulin and in transmembrane signaling (Rafaeloff et al., 1989). These observations suggested therefore that sequence 243–251 interacts with insulin. In the present study we have produced an antiserum to a synthetic peptide containing residues 241–251 (Table I). As expected, the antiserum, AP-II, was able to immunoprecipitate the denatured receptor $\alpha$ subunit. AP-II also precipitated the aforementioned 23-kDa fragment generated by the digestion of the labeled receptor $\alpha$ subunit with endoproteinase Glu-C. These data directly demonstrate therefore that this 23-kDa fragment contains the epitope presented by amino acid sequence 241–251 and thus substantiates our original assignment of the 23-kDa fragment to the cysteine-rich region. AP-II also precipitated denatured $\alpha$ subunits of insulin receptors of mouse, rat, rabbit, and guinea pig (C. C. Yip, unpublished data), indicating that amino acid sequence 241–251 is conserved in the insulin receptor of these animals. AP-II however did not react with the denatured receptor $\alpha$ subunit of the mutant receptor (C. C. Yip and I. D. Goldfine, unpublished data) in which sequence 243–251 has been mutated (Rafaeloff et al., 1989).

Waugh et al. (1989) recently reported that an insulin-labeled fragment of 55 kDa was obtained by proteolytic digestion of unreduced insulin receptor chemically cross-linked with [$^{125}$I]iodoinsulin. They obtained a fragment of similar mass by digestion of preparative amounts of unreduced insulin receptors which had not been cross-linked with insulin and found that this unlabeled fragment was recognized in immunoblots by an antiserum raised against a synthetic peptide containing sequence 242–253. It was not reported if the antiserum was able to precipitate the labeled 55-kDa fragment generated from receptors cross-linked with insulin. However, on the basis of these observations and a partial N-terminal amino acid sequence, they suggested that the region spanning residues 155–312 contained an insulin-binding site. These studies therefore are in agreement with our original assignment of the 23-kDa fragment containing an insulin-binding site as being derived from residues 205–316.

In contrast to these findings, Wedekind et al. (1989) using a photoactive biotinylated insulin analogue concluded that a part of the insulin-binding region contained sequence 20–120. This conclusion was based on a partial and tentative amino acid sequence assignment of two amino acid residues in a 14-kDa tryptic fragment. Thus, their assignment of the insulin-binding site, instead of being located in the cysteine-rich region of the receptor $\alpha$ subunit, was outside the cysteine-rich region. In preliminary experiments we have produced an antiserum against a synthetic peptide containing amino acid sequence 81–92 (C. C. Yip, I. D. Goldfine, and C. Grunfeld, unpublished data). We found that the antiserum, which was strongly reactive with the synthetic peptide, did not immunoprecipitate the labeled 23-kDa fragment (data not shown), indicating that the fragment did not contain sequence 81–92. It is possible that the biotinylated insulin photoprobe used by Wedekind et al. (1989) did not interact properly with the insulin-binding domain of the receptor because of the presence of a bulkyl lysylbiotinyl group. However, the possibility that the amino-terminal portion of the receptor $\alpha$ subunit also participates in the binding of insulin cannot be excluded.

If amino acid sequence 241–251 either is a part of the insulin-binding domain or is close to the site where insulin binds, antiserum against sequence 241–251 might be expected to have an effect on insulin binding to undenatured receptors. Antiserum AP-II, however, did not inhibit insulin binding to the receptor. These data indicate either that the antiserum was unable to recognize and interact with this sequence because of conformational restrictions or that sequence 241–251 was not part of the insulin-binding domain.

AP-II did immunoprecipitate both the denatured receptor $\alpha$ subunit and the 23-kDa proteolytic fragment of the $\alpha$ subunit (Figure 3). In contrast, it did not immunoprecipitate the undenatured receptor since receptors labeled with either of the two photoprobes were not recognized by the antiserum (Figure 4A,B). Antiserum AP-II also did not precipitate undenatured $^{125}$I-labeled receptors (Figure 5A) but precipitated these receptors after they had been denatured by reduction and alkylation (Figure 5B). These observations suggest therefore that the epitope containing residues 241–251 in the native receptor was inaccessible to the antiserum, and became accessible when the receptor was denatured.

Precipitation by AP-II of the undenatured insulin receptor that was photoaffinity labeled with the cleavable insulin photoprobe, [$^{125}$I]AZAP-insulin, was greatly increased after release of insulin with dithionite (Figure 4C). The possibility was considered that this change in immunoreactivity of the receptor was the result of treatment with dithionite per se and was independent of its action on azo cleavage of the photoprobe. However, receptors treated with dithionite remained unreactive with AP-II (Figure 5B, lanes 1 and 2). Further, dithionite treatment of receptors labeled with the noncleavable photoprobe increased their precipitation by AP-II by less than 2-fold, compared to a 10-fold increase in the case of receptors labeled with the cleavable photoprobe. Therefore, the conformational changes caused by dithionite alone were insufficient to expose sequence 241–251, and the increased immunoprecipitation of receptors photolabeled with the cleavable photoprobe after azo cleavage with dithionite was the result of removal of insulin from its binding site. These observations indicate that (1) AP-II was unable to react with receptors labeled with either photoprobe because sequence 241–251 was blocked by the insulin moiety cross-linked to the binding domain and (2) in the case of receptors labeled with the cleavable photoprobe this sequence became and remained accessible to the antiserum after removal of insulin by azo cleavage with dithionite.

The effects of dithionite on native receptors and on receptors labeled with [$^{125}$I]iodine or insulin photoprobes are interesting. Native receptors treated with this reagent lost their ability to bind insulin and as expected could not be photolabeled. It is therefore reasonable to conclude that dithionite treatment denatured the insulin receptor. The extent of receptor dena-

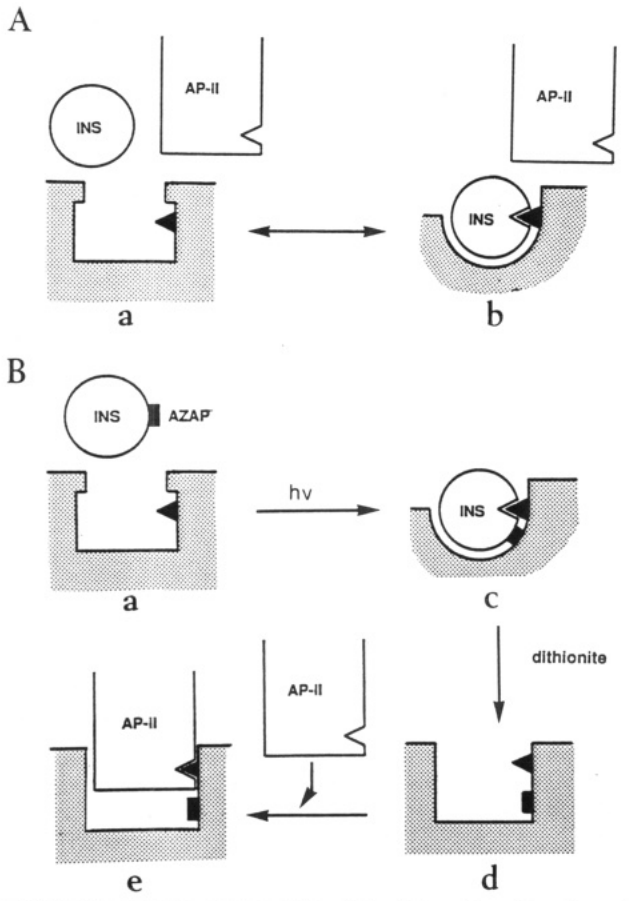


FIGURE 6: Schematic representation of the interaction of insulin and of antiserum AP-II with the insulin-binding domain of the receptor. (A) Insulin binds to the binding domain (a), which is not accessible to AP-II, to form a complex (b) in which the binding-domain assumes a new conformation induced by insulin. A conformational change in insulin is also depicted, in keeping with the suggestion that a change in the conformation of the C-terminal region of the insulin B-chain is necessary for biological activity (Nagagawa & Tager, 1987). Dissociation of insulin from the complex (b) restores the domain to its original conformation (a). The solid triangle represents the epitope containing residues 241–251. (B) The cleavable photoprobe AZAP-insulin interacts with the binding domain and induces similar conformational changes as in (A). Photolysis of the insulin–receptor complex covalently cross-links insulin to its binding domain (c). The epitope in this complex remains inaccessible to AP-II. Treatment of (c) with dithionite removes insulin through azo cleavage of the photoprobe, leaving behind the iodophenyl group covalently linked to the insulin-binding domain. The presence of the covalently linked iodophenyl group prevents the complete restoration of the binding domain to its original conformation (a) and keeps the domain in an altered conformation (d) accessible to AP-II to form an immune complex (e).

turation was demonstrated by the observation that $^{125}$I-labeled receptors treated with dithionite could no longer be precipitated by the anti-receptor serum ARS-2 (Figure 5A, compare lane 8 with lane 4). In contrast, dithionite-treated photolabeled receptors were precipitated, though less than the untreated photolabeled receptors, by the antiserum ARS-2 (Figure 4). Thus the presence of insulin photo-cross-linked to the receptor seems to have protected the receptor to some extent against the denaturing effect of dithionite so that the receptor could still be recognized by the antiserum ARS-2. We do not know how this may occur. However, it may indicate that the structure of the insulin–receptor complex, more specifically the photolabeled receptor, is less sensitive than the free receptor to the denaturing effect of dithionite.

We propose therefore (Figure 6) that sequence 241–251 is part of the insulin-binding domain and that in the native undenatured insulin receptor this domain is in the form of a pocket or crevice and is accessible to insulin but is inaccessible to antibody molecules. Upon insulin binding, the receptor undergoes a conformational change such that the domain becomes accessible to antibody molecules. In this regard it is relevant to note that undenatured photolabeled receptors are precipitated by anti-insulin serum (C. C. Yip, unpublished observation), indicating that insulin bound or cross-linked to the binding domain is accessible to antibody molecules. Dissociation of insulin from the receptor restores the binding domain to its original conformation. However, in the case of photoaffinity labeling with the cleavable insulin probe, the altered conformation of the binding domain induced by the insulin photoprobe was at least partly maintained after removal of insulin by dithionite treatment. This is most likely due to the presence of the iodophenyl group released from the AZAP-insulin and covalently linked to the peptide backbone of the insulin-binding domain. The formation of covalent bonds and the presence of the iodophenyl group may act to prevent the restoration of the domain to its native conformation, making it possible for the antibody to react with sequence 241–251. In the case of receptors photoaffinity labeled with the noncleavable MAB-insulin, sequence 241–251 remained blocked by insulin as it could not be cleaved from this probe by dithionite. Nevertheless, treatment of receptors labeled with the noncleavable MAB-insulin with dithionite did result in some precipitation of the labeled receptors. This could be the result of the denaturing effect of dithionite on the receptor as evident from the loss of insulin-binding activity in receptors treated with this reagent. However, we cannot eliminate the unlikely possibility that the iodophenyl group left cross-linked to the receptor after azo cleavage produced a unique stress on the structure of the receptor to expose sequence 241–251. Unfortunately, it is not possible to carry out control experiments in which an iodophenyl group without insulin can be cross-linked to the same site on the receptor to test this possibility.

Previous studies (Herrera et al., 1986; Perlman et al., 1989) using antibodies raised against synthetic peptides corresponding to different regions of the two subunits of the insulin receptor suggest that the conformation of the subunits in the extracellular domain of the insulin receptor may be rigid, whereas the cytoplasmic domains of the $\beta$ subunit undergo conformational changes upon autophosphorylation. However, insulin-induced conformational changes in the insulin receptor $\alpha$ subunit has been inferred from several studies. Wilden and Pessin (1987) observed that insulin receptor kinase exhibited a differential sensitivity to thiol and oxidizing agents in the absence and presence of insulin and suggested that some critical and essential thiol group(s) of the receptor have become more susceptible to these reagents after insulin binding. Forsayeth et al. (1987) reported that several monoclonal antibodies to the insulin receptor $\alpha$ subunit inhibited insulin binding and mimicked insulin activity. The binding of these antibodies to the insulin receptor was not inhibited by insulin. Moreover, these antibodies slowed the dissociation of bound insulin from its receptor. These observations indicate that insulin-mimetic monoclonal antibodies induced conformationl changes in the insulin receptor subunit and suggest that insulin may also induce similar changes. The extracellular insulin-binding domain of the receptor, obtained as an expression product of CHO cells transfected with cDNA encoding the entire insulin receptor ectodomain, was found to bind insulin with high affinity and to display a gross structural change observable by electron microscopy (Johnson et al., 1988),

although the stuctural change was induced also by epidermal growth factor. In the present study, by photoaffinity labeling of the insulin-binding domain with a cleavable photoprobe and using an antiserum against a sequence in this domain, we obtained evidence which strongly supports our hypothesis that insulin induces a conformational change in the insulin receptor $\alpha$ subunit as illustrated in Figure 6. Direct proofs of this hypothesis would have to await the X-ray crystallographic comparison between free receptor and insulin–receptor complex. Less direct methods would involve the development of conformation-sensitive antibodies which could detect changes in the insulin-binding domain as the result of insulin binding. However, this would not be possible without some knowledge of the location of the binding site. Our present observations made with the antibody AP-II provide such an approach.

## Added in Proof

Since the acceptance of this paper, Gustafson and Rutter (1990) have shown by chimera receptors that sequence 230–285 in the cysteine-rich domain of insulin receptor is the primary determinant of hormone binding specificity.

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