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Conformational Changes in the α- and β-Subunits of the Insulin Receptor Identified by Anti-peptide Antibodies*

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The structure of the insulin receptor was studied with polyclonal antibodies obtained from rabbits which were immunized with synthetic peptides having a sequence identity to three regions of the α-subunit and five regions of the β-subunit. None of the α-subunit antibodies including α-Pep8 (residues 40–49 (Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., and Ramachandran, J. (1985) *Nature* 313, 756–761), α-Pep7 (12 amino acid C-terminal extension (Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiar, F., Kan, Y. W., Goldfine, I. D., Roth, R. A., and Rutter, W. J. (1985) *Cell* 313, 747–758)), or α-Pep6 (residues 1-7, 9) immunoprecipitated the human insulin receptor solubilized from IM-9 lymphocytes; however, α-Pep8 immunoprecipitated the dithiothreitol-reduced receptor. Antibodies prepared against the N terminus of the β-subunit (α-Pep5, residues 780–790) and the ATP binding site (α-Pep3, residues 1013–1022) did not react with the intact receptor under any conditions; however, antibodies to the C terminus of the β-subunit (α-Pep1, residues 1314–1324) and to the juxta-membrane region (α-Pep3, residues 952–962) immunoprecipitated the solubilized receptor in both its phosphorylated and nonphosphorylated forms. In contrast, the antibody reactive with the regulatory region of the β-subunit which contains the major autophosphorylation sites (α-Pep2, residues 1143–1154) only precipitated the phosphorylated form. Thus the conformation of the extracellular domain of the receptor is rigid and stabilized by disulfide bonds, whereas several regions of the intracellular domain are accessible to antibodies and undergo conformational changes during autophosphorylation.

Over the past 10 years considerable information has been gained regarding the major structural and functional features of the insulin receptor (1, 2). The insulin receptor precursor is encoded by a single gene (3, 4). During synthesis it dimerizes and is cleaved to form a heterotetramer consisting of two α- and two β-subunits (1, 5). Both subunits are *N*-glycoslyated (6), and some evidence indicates that the β-subunit may also be *O*-glycosylated (7). Both subunits apparently contain covalently attached fatty acids (8). Based on surface labeling and the deduced amino acid sequence, the α-subunit is entirely extracellular, consistent with its role as the insulin-binding domain (3, 9, 10). Like many membrane receptors, the α-subunit contains a cysteine-rich region but few if any free sulfhydryl groups suggesting that it is stabilized by multiple intrasubunit disulfide bonds; easily reduced disulfide bridges exist between α-subunit dimers (11). The β-subunit is a transmembrane protein (3, 10). Its extracellular N terminus contains 4 cysteine residues, which probably form disulfide bonds with the 4 cysteine residues in the C terminus of the α-subunit (12); these intersubunit bridges are relatively resistant to reduction. The intracellular domain of the β-subunit contains at least four functional regions: the juxta-membrane domain which may play a role in signal transduction (centered around Tyr-960) (13), the ATP-binding site (centered around Lys-1018) (14), and two autophosphorylation domains (centered around Tyr-1146, -1150, and -1151, and around Tyr-1316 and -1322) (15). Tyrosyl residues 953 and 960 do not appear to be major sites of autophosphorylation, although mutation of Tyr-960 alters the ability of the receptor to produce a normal biological signal (13). A schematic model of the insulin receptor illustrating these features is shown in Fig. 1.

The conformation of the insulin receptor tetramer and the relation between structure and signal transmission is unclear. Tyrosyl phosphorylation is thought to mediate signal transmission, but other mechanisms relying mainly on conformational changes or noncovalent interactions may play important roles. The use of specific antibodies may contribute to our understanding of the conformation of the insulin receptor. We have produced eight different polyclonal antibodies, each directed against a small amino acid sequence in the human insulin receptor. Five of the segments were in the β-subunit and three were in the α-subunit. Based on the ability of these antibodies to react with the intact or partially reduced receptor and the autophosphorylated receptor, we have deduced a model suggesting that the conformation of the extracellular domain is rigid and stabilized by disulfide bonds, whereas portions of the intracellular domain are flexible and undergo conformational changes during autophosphorylation.

EXPERIMENTAL PROCEDURES AND RESULTS[1]

DISCUSSION

The amino acid sequences used to prepare anti-insulin receptor antibodies are shown in Table I. The first set of

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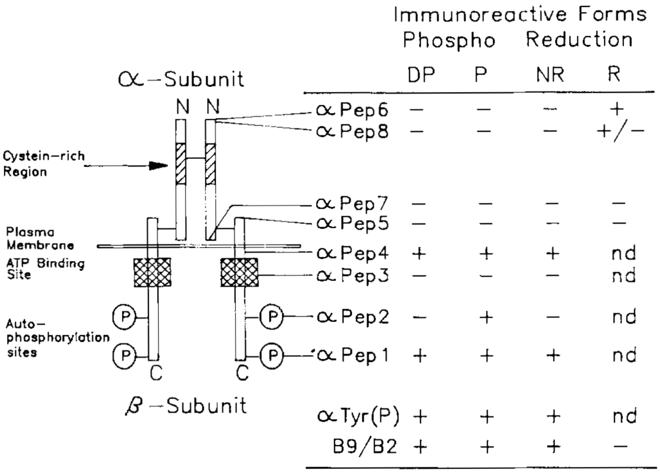
[1] Portions of this paper (including "Experimental Procedures," "Results," and Figs. 2-7) are presented in miniprint at the end of this paper. The abbreviations used are HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KLH, keyhole limpet hemocyanin;

TABLE I

Peptides used for antibody production

This numbering sequence was described by Ullrich *et al.* (3) and is based on the position of the amino acids in the precursor of the human insulin receptor. The C-terminal extension was described by Ebina *et al.* (4).

No.	Subunit	Domain	Residues	Sequence
				Intracellular (β-subunit)
1	β	C terminus	1314–1324	Arg-Ser-Tyr-Glu-Glu-His-Ile-Pro-Tyr-Thr-His
2	β	Regulatory site	1143–1152	Arg-Asp-Ile-Tyr-Glu-Tyr-Asp-Tyr-Tyr-Arg
3	β	ATP-binding site	1013–1022	Thr-Arg-Val-Ala-Val-Lys-Thr-Val-Asn-Glu
4	β	Juxta-membrane	952–962	Leu-Tyr-Ala-Ser-Ser-Asn-Pro-Glu-Tyr-Leu-Ser
				Extracellular (β-subunit)
5	β	N terminus	780–790	Arg-Ile-Glu-Leu-Gln-Ala-Cys-Asn-Gln-Asp
				Extracellular (α-subunit)
6	α	N terminus	1–7, 9	His-Leu-Tyr-Pro-Gly-Glu-Val-Lys-Pro
7	α	C terminus	Extension	Lys-Thr-Ser-Ser-Gly-Thr-Gly-Ala-Glu-Asp
8	α	N terminus	40–49	Lys-Thr-Arg-Pro-Glu-Asp-Phe-Arg-Asp-Leu



	Immunoreactive Forms			
	Phospho		Reduction	
	DP	P	NR	R
α Pep6	−	−	−	+
α Pep8	−	−	−	+/−
α Pep7	−	−	−	−
α Pep5	−	−	−	−
α Pep4	+	+	+	nd
α Pep3	−	−	−	nd
α Pep2	−	+	−	nd
α Pep 1	+	+	+	nd
α Tyr(P)	+	+	+	nd
B9/B2	+	+	+	−

FIG. 1. **Schematic representation of the insulin receptor and summary of immunological reactions.** The model depicts the insulin receptor tetramer and indicates the location of the peptides used as antigens. The tabulated summary of results indicates whether the receptor reacted with the dephospho- (*DP*) or phospho- (*P*) forms of the receptor and before (*NR*) or after (*R*) reduction of disulfide bonds. *nd* signifies that the measurement was not done.

peptides corresponds to intracellular regions of the β-subunit of the human insulin receptor. Peptides 1 and 2 contain the major tyrosyl autophosphorylation sites (15), peptide 3 is from the ATP-binding site (14), and peptide 4 contains Tyr-960 which may play a role in signal transduction (13). Peptide 5 is located at the N terminus of the β-subunit which is extracellular based on the predicted amino acid sequence of the insulin receptor (3). The third set of peptides are located in the α-subunit. Peptide 7 contains the 12-amino acid extension of the C terminus of the α-subunit reported by Ebina *et al.* (4) and Whittaker *et al.* (24); this region was not found in the cDNA cloned by Ullrich *et al.* (3). Peptides 7 and 8 are derived from the N terminus common to all α-subunit sequences reported.

The specificity of each anti-peptide antibody toward the insulin receptor is summarized in Fig. 1. All eight peptides produce specific polyclonal antibodies in rabbits (Fig. 2), but only three of these antibodies recognize the intact solubilized human insulin receptor through recognition of the intracellular portion of the β-subunit (Fig. 3). Two of these three antibodies, one to the juxta-membrane region (α-Pep4, including Tyr-953 and Tyr-960) and one to the C terminus (α-Pep1, including Tyr-1316 and Tyr-1322) recognize the receptor in all forms: dephosphorylated, phosphorylated, unoccupied and occupied with insulin, iodinated, and affinity-labeled with ^{125}I-insulin. The antibody directed toward the ATP binding site (α-Pep3, including Lys-1018) between the juxta-membrane region and the regulatory region fails to recognize the receptor in either its phospho- or dephospho-form.

The antibody which recognizes the regulatory domain of the insulin receptor (α-Pep2, including Tyr-1146, -1150, and -1151) immunoprecipitates only the autophosphorylated form; insulin binding alone is insufficient to cause immunoprecipitation (Figs. 4 and 5). These results are similar to data reported by Herrera *et al.* (25, 26) and suggest that the β-subunit of the insulin receptor undergoes a conformational change following autophosphorylation. During *in vitro* autophosphorylation, Tyr-1146, -1150, and -1151 in the β-subunit are phosphorylated (15), suggesting that tris-phosphorylation in this region does not significantly block recognition by α-Pep2. It is possible that the phosphorylated receptor has a higher affinity for the antibody than the dephospho-form, but this explanation is unlikely as the antibody was prepared against a nonphosphorylated peptide. Moreover, *in vivo* autophosphorylation which occurs predominantly on two residues, Tyr-1146 and either Tyr-1150 or -1151 (15), also mediates immunoprecipitation by α-Pep2 (Fig. 6). Thus bis-phosphorylation of the regulatory region is sufficient to cause the conformational change in the β-subunit. Furthermore, autophosphorylation of Tyr-1316 and -1322 is minor *in vivo* (15) suggesting that phosphorylation of the C terminus does not play a major role in the conformational changes in the regulatory region.

None of our anti-peptide antibodies to the α-subunit or the extracellular domain of the β-subunit recognize the intact receptor in the presence or absence of insulin or after iodination, phosphorylation, or affinity labeling. They are unreactive even though these antibodies have a higher titer on the synthetic peptides than the antibodies which bind to the intracellular regions. Although the peptide antigens were selected because they did not contain potential glycosylation sites, these regions of the α-subunit may be sterically blocked by nearby carbohydrate moieties. However, a marked increase in reactivity of the receptor to α-Pep8 and a weak reactivity to α-Pep6 (the N terminus of the α-subunit) occurs after reduction of the receptor *in vitro* with dithiothreitol (Fig. 7). Since α-Pep8 strongly reacts with the receptor after mild reduction which cleaves the disulfide bridges between α-sub-

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PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; WGA, wheat germ agglutinin (wheat germ agglutinin agarose was from Vector); α-PY, anti-phosphotyrosine antibody.

unit dimers (11), it is possible that a major contact point between α-subunit molecules occurs near amino acid residues 40–49. Thus the lack of reactivity of the α-subunit antibodies may be attributed to a highly structured and rigid extracellular domain caused by considerable disulfide bonding.

The α-Pep7 reacts with the amino acid sequence found at the C terminus of the Ebina *et al.* sequence (4). Because this region is absent from the amino acid sequence of Ullrich *et al.* (3), α-Pep7 may serve as a probe to detect the presence of receptors that contain the 12-amino acid extension in the C terminus of the α-subunit. This antibody did not recognize any form of the insulin receptor that we prepared from IM-9 lymphocytes. Whether it works during immunoblots is unknown. The lack of immunoprecipitation may be due to poor binding of αPep-7 to the denatured receptor molecules; however, we provisionally conclude that few or none of the receptors in IM-9 lymphocytes contain the 12-amino acid extension of the α-subunit.

Our data are largely consistent with other immunological data on the insulin receptor. When monoclonal antibodies are produced to the intact receptor, the large majority is directed toward the intracellular domains of the β-subunit (27). Although monoclonal antibodies occasionally occur to the α-subunit (28), the exact peptide region to which they bind has not yet been determined. Autoantibodies to the receptor occur in patients with the Type B syndrome of insulin resistance and acanthosis nigricans (29). As most of these antibodies inhibit insulin binding, they presumably bind to the α-subunit of the receptor. However, these antibodies appear to recognize three-dimensional features of the receptor since they do not immunoprecipitate the receptor after reduction (Fig. 7).

Herrera *et al.* (25, 26) have prepared antibodies to synthetic peptides of β-subunit to study receptor structure. As in our work, they found that antibodies to the regulatory domain around Tyr-1150 immunoprecipitated the receptor only after autophosphorylation (25). However, their antibody to the juxta-membrane domain (AbP4) inhibited insulin-stimulated phosphotransferase when it was allowed to bind before autophosphorylation, whereas it had no effect on kinase activity when it was added after autophosphorylation. Our antibody to this region (α-Pep4) had no inhibitory effect on autophosphorylation. The peptide used by Herrera *et al.* (25, 26) to generate AbP4 was 5 amino acids longer than ours, suggesting that residues 963–967 may play a critical role in receptor function.

In summary, our data suggest that the extracellular domain of the insulin receptor has a rigid and ordered structure with the N termini of both subunits restricted sterically in the three-dimensional structure. By contrast, the juxta-membrane and C-terminal regions of the intracellular domain of the intact β-subunit are readily accessible to antibodies. The regulatory region containing a cluster of three autophosphorylation sites is accessible to its cognate antibody only after autophosphorylation. Further insights into the three-dimensional structure of the receptor awaits having sufficient quantities of material for a more direct analysis. The current data, however, provide support for an allosteric model of receptor activation. Thus, one of the consequences of autophosphorylation in the Tyr-1150 domain is to activate the phosphotransferase in the β-subunit; the other may be to cause a conformational change which initiates a signal transmission through an unknown mechanism.

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Supplementary Material to

CONFORMATIONAL CHANGES IN THE α- AND β-SUBUNITS OF THE INSULIN RECEPTOR IDENTIFIED BY ANTI-PEPTIDE ANTIBODIES

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EXPERIMENTAL PROCEDURES

Materials---Tissue culture plastic-ware was obtained from NUNC. F-12 and RPMI 1640 tissue culture medium and fetal bovine serum were from GIBCO. The [γ-^{32}P]ATP, Na[^{125}I], [^{32}P]orthophosphate and [^{125}I]protein A were from New England Nuclear, and [^{125}I](B26)insulin was from Amersham. HEPES, tris, aprotinin, phenylmethylsulfonyl fluoride, lactoperoxidase, N-acetylglucosamine, and dithiothreitol were from Sigma, and other reagents were from Fisher. Reagents for SDS-PAGE and the Bradford protein assay were from Bio-Rad. Wheat germ agglutinin-agarose was from Vector Laboratories, and protein A-agarose was from Pierce. Synthetic peptides (Table I) corresponding to the sequence of eight separate regions of the human insulin receptor precursor (3) were purchased from Peninsula Laboratories. The anti-phosphotyrosine antibody was prepared as previously described (16). CHO/HIRC$_1$ cells were obtained by transfecting Chinese hamster ovary cells with an expression vector containing the wild-type human insulin receptor as previously described (13).

Production of anti-peptide antibodies---Polyclonal antibodies were produced against specific peptide sequences of the human insulin receptor as described by Goren et. al. (17). The peptides were coupled to Keyhole limpet hemocyanin (Calbiochem) with bromoacetyl bromide (Aldrich) (16). The peptide-KLH conjugate (1 mg in 100 μl phosphate buffered saline) was mixed with 0.9 ml of complete Freund's adjuvant (DIFCO), sonicated briefly and injected intradermally into New Zealand white rabbits. Rabbits were boosted subsequently at 21 day intervals with 1 mg of peptide-KLH conjugate mixed with incomplete Freund's adjuvant (DIFCO). Serum was harvested 14 days after each injection and frozen at -20°C before purification (17).

Rabbit serum was screened for anti-peptide IgG by specific binding to the cognate peptide immobilized on polyvinyl multiwell plates (Costar). Each multiwell was incubated with 50 μl of PBS containing 1 mg/ml peptide and then washed with PBS containing 1% bovine serum albumin (BSA, Sigma) to remove unbound peptide and block unoccupied binding sites. Rabbit sera diluted 1:10, 1:100, 1:500 and 1:1000 were incubated with each peptide at 22°C for 1 h. Unbound proteins were removed by washing with PBS containing 1% BSA. Bound IgG was detected by incubating each well with PBS containing 1% BSA and 10,000 CPM of [^{125}I]protein A, followed by washing and counting each well in a gamma counter.

Sera exhibiting the highest titer were purified by affinity chromatography on the immobilized peptide. One mg of peptide was coupled to 2 ml of Affi-gel 10 (Bio-Rad) in dimethyl sulfoxide as described in the manufacturers instructions. After the reaction, the Affi-gel was washed with PBS, and the corresponding rabbit serum (≤ 50 ml) was applied to the column. The column was washed with 50 ml of PBS at 4°C. The bound IgG was eluted from the washed column with 100 mM glycine (pH 2.5) in 0.5 ml factions, and neutralized immediately in 0.5 ml of 1 M HEPES, pH 7.4. The yield of specific antibodies varied widely among the rabbits, but the average yield was about 0.5 mg per 10 ml of serum. The antibody was diluted to 0.1 mg/ml with water and stored at -70°C.

Iodination of the extracellular domain of the insulin receptor---Human lymphocytes (IM-9) or CHO/HIRC$_1$ cells were surface-iodinated as previously described (18). IM-9 cells (about 10^7 cells) were grown in RPMI 1640 medium, collected by low-speed centrifugation and washed 3 times with cold PBS. The cells were suspended in 10 ml of cold PBS containing 2 units/ml lactoperoxidase and 10 mM β-D-glucose. Iodination was initiated by adding 0.5 mCi/ml Na^{125}I followed by 1 unit/ml glucose oxidase. Iodination was carried out at 4°C for 30 minutes with gentle mixing. The reaction was stopped by washing the cells 5 times with cold PBS.

Iodination of confluent CHO/HIRC$_1$ cells was similar, except the washing and iodination were completed on dishes. Enough iodination solution to cover the confluent cells was added to each culture dish, and the dishes were rocked gently for 30 minutes on ice (18).

Iodinated cells were incubated without or with 100 nM insulin for 2 minutes at 37°C. The cells were solubilized immediately at 4°C in 25 mM HEPES (pH 7.4) containing 1% Triton X-100, 2 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, 4 mM EDTA, 1 mg/ml aprotinin and 2 mM PMSF as previously described (18). The insoluble material was removed by ultracentrifugation, and the receptor was purified from the supernatant by affinity chromatography on WGA (19), and immunoprecipitated as described below.

Labeling the WGA-purified insulin receptor with [^{125}I]insulin by covalent cross-linking---The insulin receptor was purified on immobilized wheat germ agglutinin (WGA) from cultured human lymphocytes (IM-9) and CHO/HIRC$_1$ cells as described previously (15), and affinity labeled by cross-linking with [^{125}I]-insulin as described by Pilch and Czech (20). Aliquots of WGA-purified insulin receptor (5 μg) from either IM-9 or CHO/HIRC$_1$ cells were incubated for 16 h at 4°C with [^{125}I]insulin (0.5 μCi). Cross-linking was initiated by adding 5 mM (in dimethyl sulfoxide) disuccinimidyl suberate (Pierce) to a final concentration of 50 μM. After 15 minutes at 4°C the reaction was quenched by adding 500 mM tris to a final concentration of 50 mM. The [^{125}I]insulin:receptor complex was then used in immunoprecipitation experiments before and after autophosphorylation as described below.

Phosphorylation of the WGA-purified insulin receptor---Unlabeled WGA-purified insulin receptor was phosphorylated *in vitro* in the presence of [γ-^{32}P]ATP, whereas the receptor labeled by affinity cross-linking with [^{125}I]insulin or surface-iodination with [^{125}I]iodine was phosphorylated *in vitro* in the absence of [γ-^{32}P]ATP. Purified receptor (2-5 μg) was incubated for 1 h at 4°C in 50 mM HEPES containing 0.1% Triton X-100 and 100 nM insulin. Autophosphorylation was initiated at 22°C by adding 5 mM $MnCl_2$, 25 μM ATP, and 20 μCi [γ-^{32}P]ATP as required. Autophosphorylation was terminated by adding 0.5 ml 50 mM HEPES containing 2 mM Na_3VO_4, 0.1% Triton X-100, 100 mM NaF, 10 mM $Na_4P_2O_7$ and 4 mM EDTA, and the phosphorylated receptor was immunoprecipitated as described below.

Immunoprecipitation of insulin receptor with anti-peptide antibodies---In most of our experiments, immunoprecipitation of the radioactively labeled insulin receptor was performed as described by Goren et. al (17). Affinity purified IgG (1 μg) was added to the labeled insulin receptor (2-5 μg) and incubated at 4°C overnight. A 50 μl portion of 10% Pansorbin (Calbiochem) was added to the solution and incubated for 1 h at 4°C to precipitate the antibody complexes. The Pansorbin was washed twice with 1 ml of 50 mM HEPES (pH 7.4) containing 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 100 mM NaF and 2 mM Na_3VO_4. The receptor was eluted from the pansorbin pellet with 100 μl of Laemmli buffer containing 100 mM dithiothreitol (21). The precipitated proteins were separated by SDS-PAGE using a 7.5% resolving gel and identified by autoradiography.

Phosphorylation of the insulin receptor immobilized on protein A agarose---WGA-purified receptor (5 μg) was incubated with the indicated anti-peptide antibody for 1 h at 4°C. The immune-complex was precipitated on Protein A agarose (Pierce) (25 μl of 50% suspension). The immobilized receptor:antibody complex was washed with cold 50 mM HEPES containing 0.1% Triton X-100, and the agarose was suspended in 30 μl of 50 mM HEPES containing 5 mM $MnCl_2$ and 0.1% Triton X-100 in the absence or presence of 100 nM insulin for 1 h at 4°C. Phosphorylation was initiated by addition of 25 μM ATP containing 20 μCi [γ^{32}P]ATP, and terminated by dilution of the reaction into 500 μl of 25 mM HEPES containing 0.1% Triton X-100, 4 mM Na_3VO_4, 100 NaF, 10 mM $Na_4P_2O_7$ and 5 mM EDTA. The phosphorylated complex was washed twice with this buffer and the proteins were eluted with 100 μl Laemmli sample buffer and subjected to SDS-PAGE and autoradiography (22).

RESULTS

The specificity of receptor-peptide antibodies---The titer and specificity of the anti-peptide antibodies was determined with a solid-phase assay in which each peptide (Table I), bound electrostatically to microtiter wells, was incubated with each anti-peptide sera. Antibody binding was measured with [^{125}I]Protein A. Each anti-serum diluted 1:50 bound with highest titer to its cognate peptide, and generally showed < 10% cross-reactivity with the other peptides (Fig. 2). However, α-Pep2 bound weakly (<20%) to the other peptides suggesting that this serum contained a higher level of cross-reactivity than the others. Relative cross-reactivity for all the anti-sera was significantly lower at 1:500 dilution. Although the titers of the sera varied, α-Pep7, α-Pep8 and α-Pep3 had the highest titers. After affinity purification, 10 ng of each IgG showed > 95% specific binding. In the subsequent immunoprecipitation experiments, 1 μg of affinity-purified IgG was used.

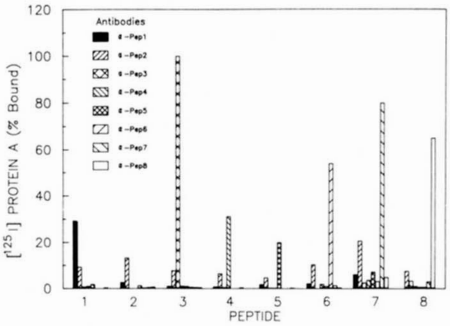

Figure 2. Specificity of anti-peptide antibodies. Each antibody indicated on the figure was incubated in duplicate on soft polyvinyl plates (96-well) coated with peptides 1 - 8 (5 pg/well). The wells were washed three times with PBS containing 5% FBS. [^{125}I]Protein A (40,000 CPM) was added to each well and incubated overnight at 4°C. The wells were thoroughly washed and all excess buffer was removed. The dried wells were excised and the bound radioactivity was measured in a γ-counter. The relative binding of protein A is shown in the figure.

Immunoprecipitation of phosphorylated receptor---The ability of the peptide antibodies to recognize the phosphorylated human insulin receptor was determined by immunoprecipitation. The insulin receptor was purified from unlabeled IM-9 lymphocytes and phosphorylated *in vitro* with [γ-^{32}P]ATP during insulin stimulation. A portion of the labeled receptor was incubated with 1 μg of each peptide antibody. As positive controls, the human polyclonal anti-insulin receptor serum B2 and rabbit polyclonal antiphosphotyrosine antibodies (α-PY) were also used; a nonimmune serum was used as the negative control. Only three of the eight anti-peptide antibodies, α-Pep1, a-Pep2 and α-Pep4, reacted with the phosphorylated receptor. These regions of the β-subunit play important functional roles as sites of autophosphorylation (15) and signal transduction (13). A similar pattern was obtained using the receptor from transfected CHO cells, and with the rodent receptor from Fao hepatoma cells (data not shown). Despite significant titers against purified peptides in the solid phase assay (Fig. 2), the antibodies to the ATP binding domain, the N-terminus of the β-subunit, and the three α-subunit regions did not immunoprecipitate the autophosphorylated receptor (Fig. 3, lanes c, e-h).

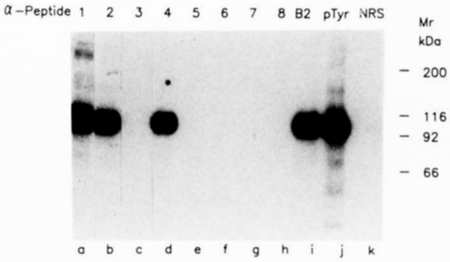

Figure 3. Phosphorylation and immunoprecipitation of WGA-purified insulin receptor from IM-9 cells. The partially purified receptor was incubated for 1 h at 4°C with 100 nM insulin. Phosphorylation was initiated by adding 25 μM ATP containing 20 μCi [^{32}P]ATP, and the solution was incubated at 22°C for 15 min. The reaction was terminated and incubated with the indicated purified anti-peptide antibodies for 2 h at 4°C. The immune complex was precipitated with Pansorbin and the phosphoproteins were separated by SDS-PAGE. An autoradiogram is shown.

Recognition of the insulin receptor by anti-peptide antibodies before and after autophosphorylation---Insulin receptor purified from CHO/HIRC$_1$ cells was phosphorylated with [γ-^{32}P]ATP during insulin stimulation, and as expected, the phosphorylated receptor was immunoprecipitated by αPep1, αPep2, αPep4 and the α-PY (Fig. 4, lanes a,e,i,m). Before autophosphorylation the insulin receptor was immunoprecipitated with α-Pep1 and α-Pep4, and was phosphorylated in the immunoprecipitate during incubation with [γ-^{32}P]ATP (Fig. 4, lanes b and c, j and k). Insulin stimulation before immunoprecipitation activated the receptor, and inclusion of insulin with the [γ-^{32}P]ATP did not further stimulate autophosphorylation (Fig. 4, lanes d and l); however, only weak phosphorylation was observed without insulin. Thus antibodies to the C-terminus (α-Pep1) and the juxta-membrane region (α-Pep4) recognize the non-phosphorylated receptor and do not inhibit autophosphorylation. In contrast, immunoprecipitates of the non-phosphorylated receptor with α-PY did not autophosphorylate, consistent with the fact that this antibody requires the presence of Tyr(P) to form an immunoprecipitate (Fig. 4, n-o). Moreover, immunoprecipitates of the non-phosphorylated receptor with α-Pep2 did not under go autophosphorylation suggesting that autophosphorylation is required for immunoprecipitation or that this antibody inhibited autophosphorylation.

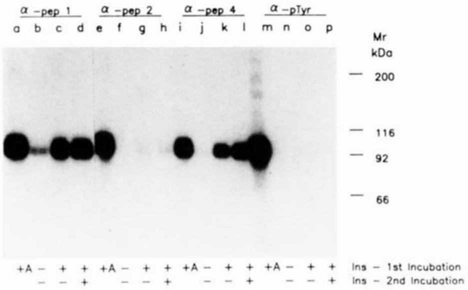

Figure 4. Autophosphorylation of the immobilized insulin receptor-antibody complex. WGA-purified insulin receptor from CHO/HIRC$_1$ cells was phosphorylated with [γ-^{32}P]ATP in the presence of 100 nM insulin and then immunoprecipitated from the reaction with the indicated antibody (lanes a,e,i,m). In separate reactions, the receptor was incubated without insulin (lanes b,f,j,n) or with 100 nM insulin (lanes c,d,g,h,k,l,o,p). Before autophosphorylation, the receptor was immunoprecipitated by addition of the indicated antibody followed by protein A agarose. The immune-complex was then incubated with [γ-^{32}P]ATP in the absence (lanes b,c,f,g,j,k,n,o) or presence (d,h,l,p) of 100 nM insulin. The phosphoproteins were reduced with 100 mM DTT and separated by SDS-PAGE.

To establish the requirement of autophosphorylation for recognition of the receptor by α-Pep2, we labeled purified receptor with [^{125}I]insulin and immunoprecipitated it before and after a 10 min incubation with 25 μM ATP. Before autophosphorylation, the affinity-labeled insulin receptor was immunoprecipitated by αPep1 and αPep4, but not by αPep2 or by α-PY (Fig. 5, lanes a,c,e,g). In contrast, after autophosphorylation αPep2 immunoprecipitated the labeled receptor without affecting immunoprecipitation by αPep1 or αPep2 (Fig. 5, lanes b,d,f). The α-PY also immunoprecipitated the affinity-labeled receptor after autophosphorylation confirming that it contained newly formed Tyr(P) residues (Fig. 5, lane h). The preferential immunoprecipitation of the autophosphorylated receptor by αPep2 was unexpected as this antibody was prepared against a dephosphorylated peptide. Thus our data suggest that residues 1143-1152 are inaccessible to the antibody before autophosphorylation, but become accessible after autophosphorylation probably as a result of a conformational change.

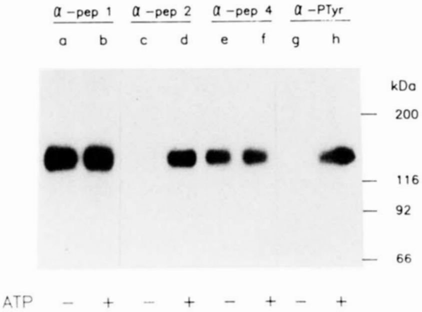

Figure 5. Immunoprecipitation of [^{125}I]insulin-labeled receptor with anti-peptide antibodies. WGA-purified insulin receptor was labeled by covalent cross-linking to [^{125}I](B26)insulin. The labeled receptor was divided into two portions and incubated without (-) or with (+) 25 μM ATP for 10min at 22°C. The reaction mixture was incubated with the indicated antibodies and the precipitated proteins were separated by SDS-PAGE.

The effect of in vivo autophosphorylation on antibody recognition---We have previously shown that the sites of tyrosine autophosphorylation of the insulin receptor *in vitro* and *in vivo* are similar, whereas the stoichiometry of phosphorylation is significantly different (15). *In vitro*, nearly 80% of the receptors are phosphorylated in the regulatory region at Tyr-1146, Tyr-1150 and Tyr-1151, and in the C-terminus at Tyr-1316 and Tyr-1322; however, *in vivo* only two of these five residues are predominantly phosphorylated: Tyr(P)-1146 and either Tyr(P)-1150 or Tyr(P)-1151. In addition, the receptor is phosphorylated *in vivo* on seryl and threonyl residues (19). To determine if these differences affect the conformational change of the receptor that is detected with α-Pep2, CHO/HIRC$_1$ cells were surface-iodinated and stimulated *in vivo* with 100 nM insulin before solubilization and immunoprecipitation. In the absence of insulin stimulation, α-Pep1 immunoprecipitated the α- and β-subunit of the insulin receptor, whereas α-Pep2 and α-PY did not (Fig. 6, lanes a,c,e). After insulin stimulation, all three antibodies recognized the labeled receptor (Fig. 6, lanes b,d,f). Thus, the limited tyrosyl autophosphorylation of the β-subunit in the intact cell causes a similar conformational change as seen *in vitro*.

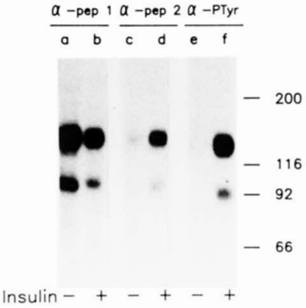

Figure 6. The effect of autophosphorylation *in vivo* on immunoprecipitation of the insulin receptor. CHO/HIRC$_1$ cells were surface-iodinated and then incubated without (-) or with (+) 100 nM insulin for 2 min at 37°C. The insulin receptor was extracted from the cells and divided into 3 equal portions for incubation with α-Pep1, α-Pep2, and α-PY. The precipitated protein were separated by SDS-PAGE.

Effects of receptor occupancy and subunit reduction on antibody recognition---As the α-subunit is cysteine-rich, it is possible that some of the receptor domains containing the peptide sequences are sterically inaccessible to the peptide antibodies due to a rigid tertiary structure. Moreover, occupancy of the insulin binding site may affect conformation and antibody recognition. To examine these possibilities, the α-subunit of the receptor purified from IM-9 lymphocytes was affinity-labeled with [^{125}I](B26)insulin by cross-linking. Before reduction, the labeled receptor was precipitated by two different human polyclonal anti-insulin receptor sera B2 and B9 (Fig. 7, lanes g and i) (23). However, as noted above, none of the antibodies to the α-subunit domains precipitated the intact labeled receptor (Fig. 7, lanes a,c,e). In an attempt to relax the conformation of the α-subunit, disulfide bonds of the affinity-labeled receptor were reduced at 22°C with 5 mM DTT for 5 min, and free sulfhydryl groups were blocked with 12 mM N-ethylmaleamide. In this reduced form, both human polyclonal antibodies lost their ability to immunoprecipitate the receptor (Fig. 7, lanes h and j) suggesting that the tertiary structure was important for their recognition. In contrast, α-Pep6 and α-Pep8 which recognize adjacent regions of the N-terminus of the α-subunit immunoprecipitated the reduced receptor (Fig. 7, lanes b and f); α-Pep8 immunoprecipitated with high efficiency. The α-Pep7 which recognizes the C-terminal extension of the α-subunit did not immunoprecipitate the reduced receptor (Fig. 7, lane d).

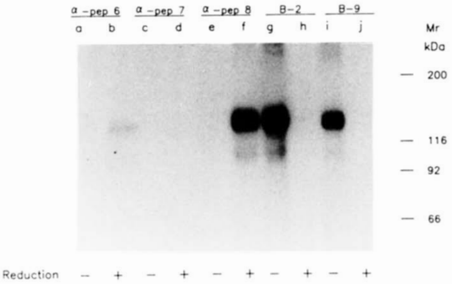

Figure 7. Immunoprecipitation of [^{125}I]insulin-labeled receptor before and after reduction of disulfide bonds. WGA-purified insulin receptor (5 μg) from IM-9 cells were cross-linked to [^{125}I](B-26)insulin. Disulfide bonds in the labeled receptor were reduced during incubation of the receptor with 5 mM DTT for 5 min at 22°C. The reaction was terminated with 12 mM NEM. The reduced receptor was incubated with the indicated α-subunit antibody, overnight at 4°C. Immunoprecipitation and SDS-PAGE were performed as described in Figure 2.