Insulin Binding to Its Receptor Induces a Conformational Change in the Receptor C-Terminus†

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ABSTRACT: Antibodies against peptides corresponding to sequences in the C-terminus of the insulin receptor β-subunit were used to approach the putative role of this receptor domain in signal generation. Two sequences were chosen and correspond to peptide C1, comprising amino acids 1309–1326, and peptide C2, comprising amino acids 1294–1317. The two antibodies produced distinct immunoprecipitation patterns as a function of the insulin receptor form and recognized changes in the insulin receptor molecule induced by ligand binding and autophosphorylation. Both antipeptides, anti-C1 and anti-C2, showed an important decrease in their recognition capacity for the receptor occupied by insulin when compared to the empty receptor. Further, anti-C1 had a lower affinity for the phosphorylated receptor compared to the unphosphorylated receptor and failed to recognize a fraction of the phosphoreceptor population. In contrast, anti-C2 had similar affinities for the phosphorylated and unphosphorylated receptors but was unable to interact with part of the unphosphorylated receptors. Finally, using immunoblotting of the receptor to analyze the denatured molecules, we showed that the phosphorylation-induced changes detected by anti-C1 are retained, suggesting that they are likely not of a conformational nature. In contrast, the insulin-induced changes in the receptor molecule disappear with receptor denaturation which points to their reversible nature. We conclude from these data that (i) antipeptides against the receptor C-terminal sequence are able to distinguish between phosphorylated and unphosphorylated receptor forms and (ii) binding of insulin to its receptor leads to a reversible, phosphorylation-independent, and possibly conformational change at the level of the receptor C-terminal domain.

Insulin induces its biological effects through interaction with a specific membrane receptor, a glycoprotein consisting of two α- and two β-subunits linked together to form a heterotetramer. The α-subunit is entirely extracellular and contains the insulin binding site. The β-subunit has an extracellular domain linked to the α-subunit by disulfide bonds, a transmembrane region, and a cytoplasmic domain which contains an insulin-inducible tyrosine kinase (Kahn, 1985; Gammeltoft & Van Obberghen, 1986; Rosen, 1987; Yarden & Ullrich, 1988). The insulin receptor belongs to the tyrosine kinase receptor family that comprises, for example, epidermal growth factor (EGF),[1] PDGF, and IGF I-receptors. After insulin binding to its specific receptor on the target cell, a wide array of biological responses is initiated, including metabolic and mitogenic responses. The earliest insulin-evoked event identified so far is tyrosine phosphorylation of the receptor catalyzed by the receptor β-subunit kinase (Kasuga et al., 1982; Van Obberghen & Kowalski, 1982). Studies with receptors mutated at the ATP binding site (lysine-1018) (Chou et al., 1987; Ebina et al., 1987) or at tyrosine residues involved in autophosphorylation, 1150 and 1151 (Ellis et al., 1986), have demonstrated that the tyrosine phosphotransferase function of the insulin receptor is an absolute requisite for the hormone to activate the receptor signalling function in cells. How insulin binding to the α-subunit generates a signal in the β-subunit is still unclear. It is now recognized that the insulin receptor can undergo conformational changes which appear to be insulin-induced at the level of the α-subunit (Pilch & Czech, 1980; Donner & Yonkers, 1983; Waugh & Pilch, 1989) and phosphorylation-induced at the level of the β-subunit (Herrera & Rosen, 1986; Perlman et al., 1989).

The different growth factors and hormones acting through their corresponding tyrosine kinase receptors have a distinct repertoire of biological responses (Yarden & Ullrich, 1988). The specificity of the program elicited by the tyrosine kinase receptors suggests that the catalytic domains have different substrate specificities or, alternatively, that intracellular domains recognize distinct cellular substrates for the catalytic domains. The carboxyl termini of the tyrosine kinase receptors, which display a high degree of heterogeneity in terms of primary structure among these receptors, could be such a region participating in substrate recognition, and by doing so defining receptor specificity. For the insulin receptor, very little is known about this receptor domain. Studies with deletion mutants have indicated that the carboxyl terminus does

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[1] Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IGF-I, insulin-like growth factor I; KLH, keyhole limpet hemocyanin; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; ELISA, enzyme-linked immunoadsorbent assay; FCS, fetal calf serum; PMSF, phenylmethanesulfonyl fluoride; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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not actively participate in regulation of the kinase activity itself, nor in endocytosis and recycling of receptors (McClain et al., 1988). In contrast, this receptor terminus appears to be necessary for mediation of insulin-induced metabolic effects (Maegawa et al., 1988), which could suggest that it is involved in recognition of "metabolic substrates". Assuming that the insulin receptor C-terminus is indeed involved in interaction with cellular targets or in regulation of this interaction, it is reasonable to hypothesize that this receptor domain might undergo conformational changes which regulate the target recognition function. In the present work, we have approached this question by using antibodies directed against peptides corresponding to sequences of the receptor C-terminus. The following sequences were studied: (i) peptide 1309–1326, which includes two potential autophosphorylation sites, tyrosine-1316 and tyrosine-1322; (ii) peptide 1294–1317. Using immunoprecipitation and radioimmunoassay of insulin receptors, we show that upon receptor activation, modifications in the receptor β-subunit take place which can be monitored by antipeptides to receptor. Our data are consistent with the idea that upon insulin binding conformational changes occur in the C-terminus of the receptor molecule.

Materials and Methods

Production of Antibodies to Synthetic Peptides. Peptide C1 (1309–1326) and peptide C2 (1294–1317) were coupled to keyhole limpet hemocyanin (KLH) with the heterobifunctional reagent succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) (Yoshitake et al., 1982). The peptide–KLH conjugate (400 μg of peptide) was mixed with complete Freund's adjuvant and injected intradermally into rabbits. The animals were boosted several times with the KLH-coupled peptide (100–200 μg of peptide) mixed with incomplete Freund's adjuvant, and sera were collected about 12 days after each injection. Serum obtained before the first injection was used as a control. Antibodies to peptides were detected by immunoprecipitation of the ^{125}I-peptide or by ELISA. Immunoglobulins were partially purified by chromatography on protein A–Sepharose as described previously (Baron et al., 1989) except that elution was performed with 0.1 M glycine/0.5 M NaCl, pH 2.5, neutralized immediately, and dialyzed against 20 mM Hepes, pH 7.6.

Cell Culture. HIR cells, mouse embryo fibroblasts transfected with expression plasmids encoding the human insulin receptor, were a gift of Dr. Axel Ullrich (Max-Planck Institute für Biochemie, München, Germany) (Ullrich et al., 1985). These cells express approximately 10^6 insulin binding sites per cell. They were grown in H21/F12 medium (1/1) supplemented with 10% fetal calf serum (FCS).

Partial Purification of Insulin Receptors. Receptors from HIR cells were partially purified by chromatography on wheat germ agglutinin as previously described (Van Obberghen et al., 1981). Briefly, HIR cells were solubilized for 60 min at 4 °C in HNTG supplemented with 1 mM EGTA and 1.5 mM $MgCl_2$. The supernatant from an ultracentrifugation step (60 min, 100000*g*, 4 °C) was applied to a WGA column, and the insulin receptor was eluted with *N*-acetyl-D-glucosamine in HNG containing 0.1 % Triton X-100, pH 7.6. Protease inhibitors were present throughout the procedure [20 μM leupeptin, 1.25 mM bacitracin, 100 units/mL aprotinin, and 1 mM phenylmethanesulfonyl fluoride (PMSF)].

Biosynthetic Labeling of Insulin Receptors with [^{35}S]-Methionine. Confluent cells on two 15-cm dishes (approximately 30×10^6 cells/dish) were washed twice with PBS and placed in methionine-free MEM Earle's medium containing 10% dialyzed FCS, L-glutamate, and L-[^{35}S]methionine (2.5 mCi/dish) (21). After 16 h, cells were rinsed twice with PBS and solubilized, and the insulin receptors were partially purified by chromatography as described above. These receptors are called ^{35}S-receptors.

Insulin Receptor Autophosphorylation. WGA-purified insulin receptors (200 fmol/sample) were exposed to insulin (10^{-7} M) during 45 min at 22 °C; the phosphorylation was initiated by addition of 15 μM [γ-^{32}P]ATP (2.5 mCi/mmol), 4 mM $MnCl_2$, and 8 mM $MgCl_2$. After 10 min at 22 °C, the reaction was stopped with a solution containing at final concentrations 100 mM sodium fluoride, 20 mM EDTA, and 200 μM sodium vanadate (Ballotti et al., 1989; Van Obberghen et al., 1983; Le Marchand-Brustel et al., 1988).

Insulin Receptor Immunoprecipitation. The labeled insulin receptors were incubated with the antipeptides for 3 h at 15 °C. Protein A was then added for 1 h at 4 °C to precipitate the receptor–antibody complex, and the pellet was washed twice with HNT buffer. The insulin receptors (^{32}P-receptors or ^{35}S-receptors) were eluted with Laemmli sample buffer containing 3% SDS and 5% β-mercaptoethanol (Laemmli, 1970). The samples were analyzed by one-dimensional SDS/polyacrylamide gel electrophoresis.

In some experiments, immunoprecipitation of ^{125}I-insulin–receptor complexes was performed. To this end, insulin receptors (200 fmol/sample) were first labeled by incubation with iodinated insulin (0.05 μCi, specific activity 200 μCi/μg) in a final volume of 100 μL of HN buffer with 0.1% BSA, pH 7.6. After 3 h at 15 °C, steady-state binding was attained, and antibodies were added. The immunoprecipitation was achieved by addition of protein A and quantitated by counting the pellets in a γ-counter.

Immunoassays of the Insulin Receptor. The design is based on the use of ^{35}S-receptors and the antipeptides anti-C1 and anti-C2. Two types of immunoassays were performed: (1) In some experiments, ^{35}S-receptor precipitation was competed for by one of the following unlabeled receptor preparations: (i) receptors as such ("native receptors"); (ii) receptors occupied by insulin; and (iii) receptors phosphorylated with unlabeled ATP. (2) In other experiments, quantitative immunoprecipitations were performed with native ^{35}S-receptors and ^{35}S-receptors occupied by insulin. The protocol of these immunoassays can be summarized as follows:

(1) Competition Radioimmunoassay with Unlabeled Native or Phosphorylated Receptors. Increasing amounts of unlabeled insulin receptors, phosphorylated or not, were incubated for 3 h at 15 °C with anti-C1 or anti-C2 (100 μg/mL) in HN buffer containing protease inhibitors. The partially purified ^{35}S-receptor was added at a constant concentration (300 fmol/sample), and the incubation was continued overnight at 4 °C. After addition of protein A, immune pellets were washed twice with HNT and resuspended in 50 μL of water before addition of the scintillation solution (1 mL) for counting. Nonspecific values were determined as the value obtained at the highest unlabeled receptor concentration.

For the phosphorylation of receptors with unlabeled ATP, insulin receptors (450 μL, 150 fmol/μL) were incubated with insulin (10^{-7} M) for 45 min at 22 °C. The phosphorylation was performed and stopped as described above except that 50 μM nonradioactive ATP was used and that the phosphorylation reaction was carried out for 2 h at 22 °C. In these conditions, at least 80% of the receptors were phosphorylated. As a control, native receptors were incubated with buffer and then with a solution containing ions but without ATP, and finally the stopping solution was added. These receptor solutions were then diluted for further use.

(2) *Radioimmunoprecipitations with ^{35}S-Receptor Occupied or Not by Insulin.* Partially purified ^{35}S-receptors (200 μL, 150 fmol/μL) either were preincubated with insulin (10^{-7} M) or with buffer alone in a final volume of 0.4 mL for 3 h at 15 °C. Increasing amounts of these receptor preparations were exposed to antipeptides anti-C1 and anti-C2 (50 μg/mL) in a solution supplemented or not with insulin (10^{-7} M). After 3 h at 15 °C, protein A was added for 1 h at 4 °C, and pellets were washed and resuspended as described previously.

Immunoblot of the Insulin Receptor. WGA-purified insulin receptors were exposed for 45 min at 22 °C to buffer (Figure 8, lane 1) or insulin (10^{-7} M) (lanes 2 and 3). Thereafter, the phosphorylation mixture was added to one of the receptor preparations incubated with insulin (lane 3), and the incubations were continued for 90 min at 22 °C. The reaction was stopped, and the various receptor forms were submitted to SDS/polyacrylamide gel electrophoresis under reducing conditions; the proteins were transferred to nitrocellulose as described by Towbin et al. (1979). Blocking was performed for 6 h at 25 °C with 5% milk in Tris buffer (10 mM Tris/140 mM NaCl, pH 7.5), and the nitrocellulose sheets were incubated overnight at 4 °C with anti-C1 (100 μg/mL), anti-C2 (200 μg/mL), or an anti-receptor antibody (50 μg/mL) (Ponzio et al., 1987). The sheets were washed 8 times alternatively with Tris buffer containing 0.1% Tween or 0.5% tween and 0.5 M NaCl, before addition of ^{125}I-anti-rabbit immunoglobulins for 3 h at 25 °C. After several washes, the nitrocellulose pieces were dried and autoradiographed.

The following buffers were used: HN, 50 mM Hepes/150 mM NaCl, pH 7.6; HNT, 30 mM Hepes, 30 mM NaCl, and 0.1% Triton X-100, pH 7.6; HNG, 50 mM Hepes, 150 mM NaCl, and 10% glycerol, pH 7.6; HNTG, 20 mM Hepes, 150 mM NaCl, 10% glycerol, and 1% Triton X-100, pH 7.6.

Results

Specificity of the Antipeptides Anti-C1 and Anti-C2. The specificity of antibodies anti-C1 and anti-C2 was determined by their capacity to immunoprecipitate the insulin receptor, after a prior incubation of the antibodies with the peptides (data not shown). Both antipeptides were able to immunoprecipitate the insulin receptor, which was completely prevented by the cognate peptide (10^{-5} M). No cross-reactivity with the other peptide was observed, indicating that although the two antigenic sites must be in close vicinity in the receptor C-terminus the two antibodies obtained react with distinct epitopes. When receptors from other animal species were used in immunoprecipitation assays, weak cross-reactivity with the antipeptides was seen. As a whole, these data indicate that the antibodies are apparently specific for the human insulin receptor.

Differential Reactivity of the Antipeptides with Various Insulin Receptor Forms. We first tested whether our antipeptides could immunoprecipitate the insulin receptor labeled by different methods. In these experiments, two additional antibodies were used: (i) a preimmune serum as a negative control; and (ii) a monoclonal antibody to the insulin receptor external domain (B6) to obtain maximal receptor precipitation (Gautier et al., 1986). Indeed, at appropriate concentrations, antibody B6 immunoprecipitates 100% of the receptor independently of its labeling. Three different WGA-purified insulin receptor preparations were used: (i) receptors labeled with ^{125}I-insulin for 3 h at 15 °C; (ii) receptors labeled with ^{32}P after insulin-stimulated (10^{-7} M) phosphorylation in a cell lysate system; (iii) ^{35}S-receptors obtained from cells biosynthetically labeled with [^{35}S]methionine. These differently labeled insulin receptors were incubated for 3 h at 15 °C with

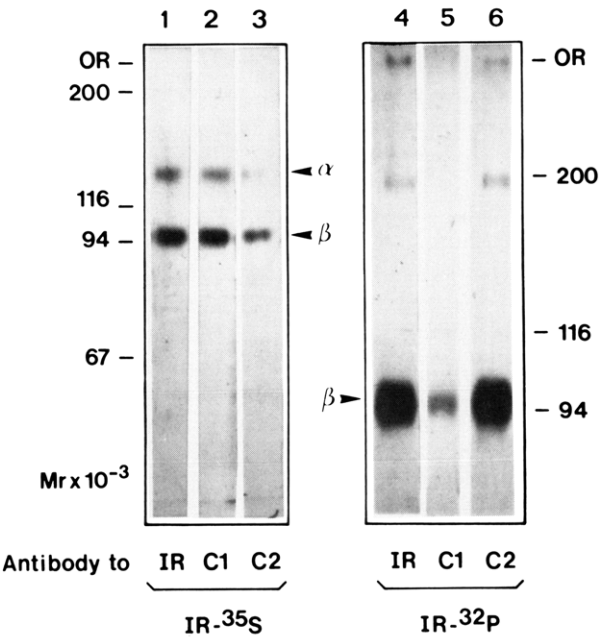

FIGURE 1: Immunoprecipitation of ^{35}S-receptors and ^{32}P-receptors. Insulin receptors were either biosynthetically labeled with [^{35}S]-methionine (lanes 1–3) or autophosphorylated in the presence of insulin as described under Materials and Methods (lanes 4–6). The labeled receptors were then incubated with antibody B6 (1/100), anti-C1 (0.5 mg/mL), or anti-C2 (0.5 mg/mL) for 3 h at 15 °C and precipitated by protein A. Samples were analyzed by SDS–PAGE, and the gel was treated with Amplify before autoradiography. The receptors were precipitated by antibody B6 (lanes 1 and 4), by anti-C1 antipeptide (lanes 2 and 5), or by anti-C2 antipeptide (lanes 3 and 6).

antibody concentrations inducing the best possible immunoprecipitation. The results obtained with the ^{35}S-receptor and the ^{32}P-receptor are shown in Figure 1. Similar to antibody B6, anti-C1 was able to provoke the immunoprecipitation of ^{35}S-receptors (lane 2 versus lane 1). In contrast, in this experiment, anti-C2 did not precipitate the total receptor population (lane 3 versus lane 1). A different immunoprecipitation pattern was obtained with the ^{32}P-receptor. Thus, while this receptor form was efficiently precipitated by anti-C2 (lane 6), anti-C1 showed a lower immunoprecipitation capacity (lane 5). This was observed with antibody concentrations similar to those used in the preceding experiment.

To ensure that the partial phosphoreceptor precipitation by anti-C1 was not due to insufficient antibody, increasing antipeptide concentrations were used (Figure 2). The immunoprecipitation observed with anti-C1 was maximal starting from 120 μg/mL (lanes 1–4), and even at this concentration, it was much lower than with anti-C2 (lanes 5–8). Further, the supernatant from precipitation with anti-C1 was submitted to a second immunoprecipitation by the antipeptide. No additional phosphoreceptor was precipitated, whereas it appeared in the final supernatant (data not shown).

When antipeptide-produced precipitation of the ^{125}I-insulin–receptor complexes was expressed as a percent of that obtained with antibody B6 (1/100), we found the following results: 44% and 42% of the liganded receptors were immunoprecipitated by anti-C1 at 0.4 and 0.6 mg/mL, respectively, and 61% and 57% of the liganded receptor were precipitated by anti-C2 at 0.4 and 0.6 mg/mL, respectively.

In summary, anti-C1 was able to immunoprecipitate the totality of the native insulin receptors but recognized only a part of the phosphorylated receptors or of the insulin–receptor complexes. Anti-C2 did not immunoprecipitate more insulin–receptor complexes than anti-C1, whereas it could recognize better the phosphoreceptor than the unphosphorylated

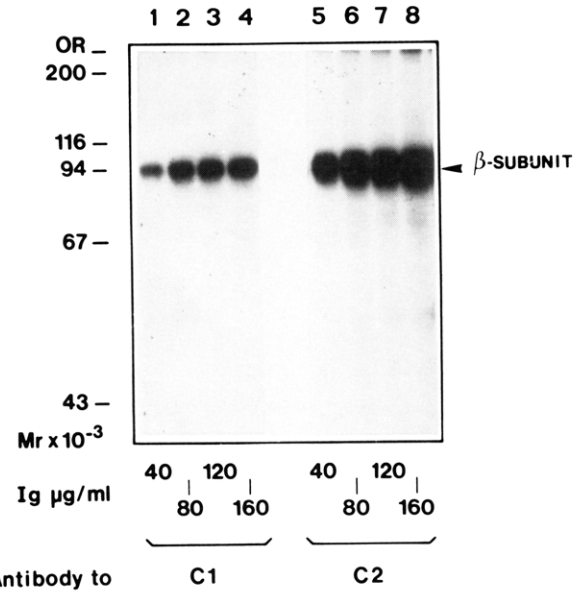

FIGURE 2: Immunoprecipitation of phosphorylated insulin receptors. Insulin receptors were incubated with 10^{-7} M insulin for 45 min at 22 °C and autophosphorylated in the presence of 15 μM [γ-^{32}P]ATP, 4 mM $MnCl_2$, and 8 mM $MgCl_2$ for 10 min at 22 °C. The reaction was then stopped, and increasing concentrations of anti-C1 or anti-C2 (40–160 μg/mL) were added for 3 h at 4 °C. After precipitation with protein A, phosphoproteins were eluted by addition of Laemmli sample buffer as described, analyzed by SDS–PAGE, and autoradiographed. Lanes 1–4, anti-C1; lanes 5–8, anti-C2.

receptor.

Immunoassays with Anti-C1 Antipeptide and Different Insulin Receptor Forms. To further analyze the ability of the antipeptides to discriminate between the different receptor forms, we performed a radioimmunoassay using ^{35}S-receptor as the tracer. This technique is based on the displacement of anti-C1- and anti-C2-induced immunoprecipitation of ^{35}S-receptors by increasing concentrations of unlabeled receptors. Note that the preparations called "phosphorylated receptors" are mixtures of receptors containing 80–90% of phosphoreceptors; likewise, "receptors occupied by insulin" correspond to a receptor mixture containing approximately 90% of occupied receptors. Consequently, the differences noted in our studies are underestimated.

(*1*) *Anti-C1 Antibody Distinguishes between Phosphorylated and Unphosphorylated Receptors.* Two pools of unlabeled receptors were used: one was phosphorylated as described under Materials and Methods; the other one was exposed to buffer only. Increasing amounts (0–3.5 pmol) of these unlabeled receptors were incubated with 100 μg/mL anti-C1. A constant amount (300 fmol) of ^{35}S-receptor was added, and samples were subjected to immunoprecipitation. As shown in Figure 3, both forms of unlabeled receptors were able to produce an inhibition of the ^{35}S-receptor precipitation. This inhibition was completely achieved with 1.6 pmol in the case of the unphosphorylated receptor, with a half-maximal precipitation of 0.7 pmol. With phosphorylated receptor, a rightward shift of the curve was observed, giving a half-maximal precipitation of 1.4 pmol, which is twice higher than with native receptor. Since more phosphoreceptor than native receptor is needed to give a similar degree of inhibition, our data indicate that anti-C1 has a lower affinity for the phosphoreceptor. The maximal inhibition was obtained with 2.7 pmol of the phosphoreceptor, but the ^{35}S-receptor precipitation was not completely abolished, indicating that an antipeptide immunoglobulin fraction fails to recognize the phosphoreceptor.

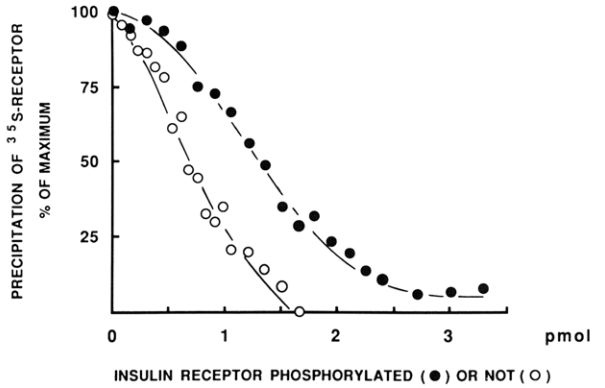

FIGURE 3: Anti-C1 has reduced affinity for phosphorylated insulin receptors. Insulin receptors were phosphorylated in the presence of insulin and 50 μM unlabeled ATP for 2 h at 22 °C, thereafter, the reaction was stopped as described under Materials and Methods. The same amount of receptors was exposed for the same length of time to a phosphorylation mixture without ATP. Increasing amounts of these phosphorylated or native receptors were incubated with 100 μg/mL anti-C1 for 3 h at 15 °C. Finally, a constant amount of ^{35}S-receptor was added for an overnight incubation at 4 °C. After protein A addition, pellets were washed twice in HNT 0.1% buffer, then resuspended in 50 μL of water/1 mL of scintillation solution, and counted. The data represented are the means of three separate experiments, each run in duplicate.

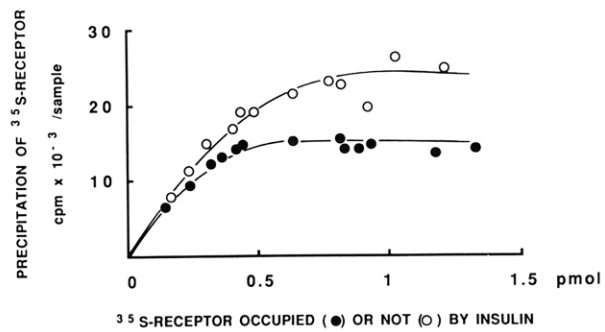

FIGURE 4: Anti-C1 discriminates between insulin receptors occupied or not by insulin. ^{35}S-Receptors were incubated with 10^{-7} M insulin or with buffer alone for 3 h at 15 °C. Increasing concentrations of these ^{35}S-receptors were then added to anti-C1 (50 μg/mL) for an overnight incubation at 4 °C. After protein A addition, the pellets were washed and resuspended as described. The data shown correspond to the means of duplicates obtained within a representative experiment.

Thus, anti-C1 showed a decreased affinity for the phosphoreceptor compared to its affinity for the native receptor and failed to recognize a fraction of the total phosphoreceptor population.

(*2*) *Anti-C1 Antibody Distinguishes between Native Receptors and Receptors Occupied by Insulin.* To evaluate the consequences of hormone binding, a radioimmunoassay similar to the one described above could not be used due to possible competition for hormone binding between the ^{35}S-receptors and the unlabeled receptors. To avoid this complication, direct binding of antipeptides to ^{35}S-receptor followed by immunoprecipitation was performed. ^{35}S-Receptors were bound or not to insulin as described under Materials and Methods and were divided into samples of increasing concentrations in the presence of anti-C1 (50 μg/mL). With the curves obtained (precipitation expressed in cpm, as a function of receptor concentration), the apparent K_d value is given at 50% of maximal precipitation, providing a plateau can be reached. As shown in Figure 4, our results allowed us to compare the relative affinities of the antibody anti-C1 for the different receptor forms. Half-maximal of the unoccupied receptor precipitation was obtained with 0.26 pmol of receptor, whereas

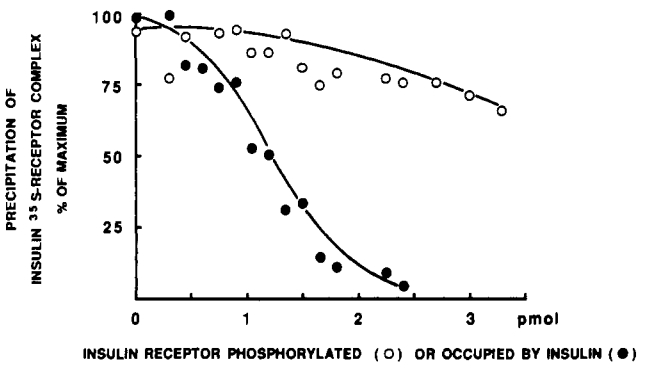

FIGURE 5: Anti-C1 distinguishes between phosphoreceptors and insulin receptor complexes. Insulin receptors were incubated with 10^{-7} M insulin for 3 h at 22 °C or with 10^{-7} M insulin for 45 min at 22 °C and then with phosphorylation mixture (50 μM unlabeled ATP) for 2 h at 22 °C. Anti-C1 (100 μg/mL) was incubated with increasing concentrations of these unlabeled insulin receptors (occupied by insulin or phosphorylated) for 3 h at 15 °C. In parallel, ^{35}S-receptor was exposed to insulin (10^{-7} M) for 3 h at 15 °C and then added to the samples at a constant concentration. The incubation was continued overnight at 4 °C, and immunoprecipitation was achieved by protein A addition. The pellets were then washed twice and counted as described in Figure 2. The data are the means of two separate experiments, each run in duplicate.

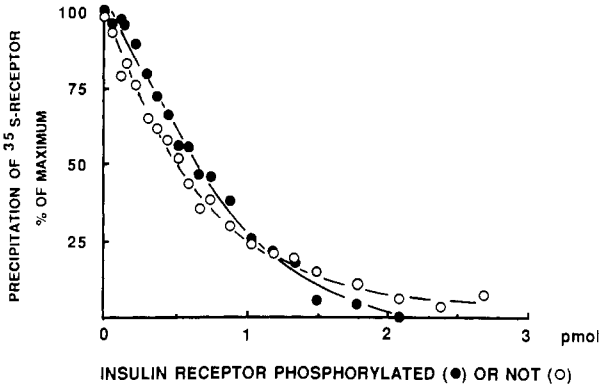

FIGURE 6: Anti-C2 has a similar affinity for the phosphoreceptor and the unphosphorylated receptor. This experiment was performed essentially as described in Figure 3 except that insulin receptors were incubated with anti-C2 (100 μg/mL). The data are the means of four experiments, each run in duplicate.

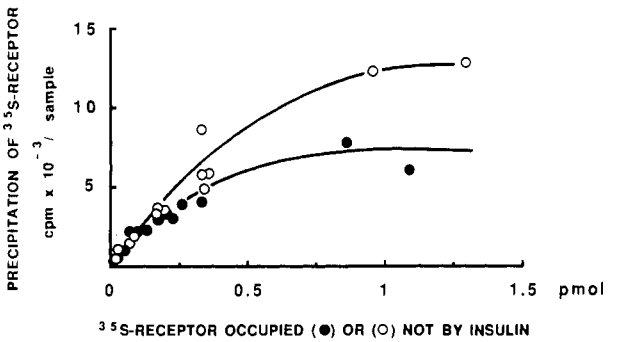

FIGURE 7: Anti-C2 discriminates between insulin receptors occupied or not by insulin. The experimental procedure was the same as in Figure 4, except that anti-C2 was added at a final concentration of 50 μg/mL. These data are the means of duplicates obtained from a representative experiment.

0.17 pmol was needed for the half-maximal precipitation of the insulin–receptor complexes, indicating that the affinity of anti-C1 for the insulin–receptor complex was slightly higher than for the unoccupied receptor. A striking difference was observed between the maximum precipitation values in the two cases, since at high receptor concentrations, i.e., 0.8–1.5 pmol, the amount of total insulin–receptor complex immunoprecipitated was only 62% of the amount of total unoccupied receptors precipitated. This suggests that remaining antibodies could precipitate receptors unoccupied by insulin, but failed to recognize the hormone–receptor complex. The observation that anti-C1 identifies only a fraction of the total receptor population after insulin binding is consistent with results obtained in experiments using ^{125}I-insulin–receptor complexes, where anti-C1 precipitated at the most 50% of the total receptor precipitated by antibody B6.

Taken together, these results demonstrate that upon insulin binding a change in the receptor C-terminus occurs, which is reflected by the loss of a class of epitopes no longer accessible to anti-C1 antipeptide.

(3) Anti-C1 Discriminates between Phosphoreceptors and Insulin–Receptor Complexes. We next wished to determine whether the anti-C1 fraction, which did not recognize the phosphoreceptor, was also responsible for the lack of recognition of the insulin–receptor complex. Unlabeled insulin receptors were phosphorylated or occupied by insulin before incubation with anti-C1 (100 μg/mL). ^{35}S-Receptors were incubated with insulin and then added to the samples and precipitated. Complete inhibition of the insulin–^{35}S-receptor complex precipitation was observed with the unlabeled insulin–receptor complex with a total displacement seen at 2.5 pmol of receptor, the half-maximal precipitation being 1.1 pmol (Figure 5). In contrast, with the phosphoreceptor only, a weak decrease in the labeled receptor precipitation was found.

We conclude from these experiments that the anti-C1 antibody fraction responsible for the insulin–receptor complex immunoprecipitation fails to recognize the phosphoreceptor form.

Immunoassays with Anti-C2 Antipeptide and Different Insulin Receptor Forms. (1) Anti-C2 Has a Similar Affinity for the Phosphoreceptor and for the Native Receptor. The ability of anti-C2 to discriminate between the unphosphorylated and the phosphorylated receptors was tested, as described for anti-C1, with increasing amounts of unlabeled receptors phosphorylated or not and a constant concentration of ^{35}S-receptors. As shown in Figure 6, the curves obtained with the phosphoreceptors and the native receptors were virtually identical, with a half-maximal precipitation occurring at 0.5–0.6 pmol of receptor. This indicates that anti-C2 had similar affinities for the two different receptor forms. Complete inhibition was not observed with the unphosphorylated receptor. To examine these results in more detail, we performed the assay using phosphorylated ^{35}S-receptors together with unlabeled phosphorylated or native receptors (data not shown). In these conditions, we also observed that inhibition with the native receptor was not complete as approximately 25% of the native receptors were not recognized by anti-C2. This is compatible with the immunoprecipitation results, where less total precipitation was seen with native receptor compared to the precipitation obtained with the phosphoreceptor (Figure 1).

(2) Anti-C2 Distinguishes between Native Receptors and Receptors Occupied by Insulin. Increasing concentrations of ^{35}S-receptors occupied or not by insulin were incubated with anti-C2 (50 μg/mL) and immunoprecipitated. The two curves, obtained with the native receptors and the insulin–receptor complexes (Figure 7), show a slight difference in anti-C2 affinity for the two receptor forms, since the half-maximal precipitation is obtained with 0.35 pmol of native receptors and with 0.2 pmol of insulin–receptor complexes. The maximal precipitation was 2-fold higher with unoccupied receptors than

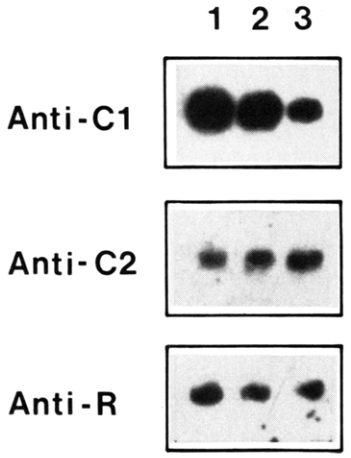

FIGURE 8: Immunoblotting of the insulin receptor. Insulin receptors were exposed to buffer (lane 1), or to insulin (lane 2), or to an insulin and phosphorylation mixture (lane 3) as described under Materials and Methods. The samples were then submitted to SDS–PAGE under reducing conditions. After electrophoresis, the proteins were transferred to nitrocellulose by Western blotting. Cellulose pieces corresponding to the receptor β-subunit were cut and incubated overnight at 4 °C with anti-C1 (100 μg/mL), anti-C2 (200 μg/mL), or polyclonal antibody to insulin receptor (50 μg/mL). ^{125}I-Anti-rabbit immunoglobulins were added for 3 h at 25 °C with several washing steps before and after. The nitrocellulose was then dried and submitted to autoradiography. Lane 1, insulin receptors exposed to buffer; lane 2, insulin receptors exposed to insulin; lane 3, insulin receptors exposed to insulin and phosphorylated.

with insulin–receptor complexes. This observation adds further support to the idea that insulin induces a change in receptor conformation, which is accompanied by a loss of epitopes, the latter being reflected by the lack of recognition of a fraction of the antipeptide population.

Immunoblotting of the Insulin Receptor Forms. To approach the question relating to the nature of the changing recognition by the antipeptides anti-C1 and anti-C2, the different insulin receptor forms were analyzed by immunoblotting (Figure 8). As a control, we used a polyclonal antibody to the insulin receptor, which recognizes the three insulin receptor forms in a comparable fashion. Similarly, antipeptide anti-C2 interacts as well with the receptor preincubated with insulin (lane 2) and the phosphorylated receptor (lane 3) as with the unmodified receptor (lane 1). In contrast, antipeptide anti-C1 recognizes much less efficiently the phosphorylated receptor (lane 3) when compared to the receptor preincubated with insulin (lane 2) and the unmodified receptor (lane 1). The results found with receptors preincubated with insulin have to be viewed with caution, since it is unlikely that the receptor retains bound ligand after SDS–PAGE. However, our data clearly indicate that the receptor change induced by ligand binding is reversible whereas the phosphorylation-induced change persists after receptor denaturation.

Discussion

Understanding the mechanism by which insulin elicits its cellular effects remains a matter of intense research. Over recent years, the following picture has emerged: insulin binds to its specific membrane receptor switching on its tyrosine kinase activity, which is a necessary step for the transduction of metabolic and mitogenic responses (Kahn, 1985; Gammeltoft & Van Obberghen, 1986; Rosen, 1987; Yarden & Ullrich, 1988). This insulin-induced cellular activation involves phosphorylation of endogenous substrates, which very likely participate in other phosphorylation/dephosphorylation reactions leading to the final hormonal effects. However, the molecular mechanism by which insulin binding to the α-subunit induces activation of the receptor β-subunit with ensuing autophosphorylation remains unclear. Molecular cloning and biochemical data have shown that the cytoplasmic part of the β-subunit contains a kinase domain, which shares strong homologies with other tyrosine kinases and which displays an ATP binding site and several autophosphorylation sites (Yarden & Ullrich, 1988). The C-terminal domain contains two autophosphorylation sites (tyrosine-1316 and -1322), which could be implied in regulation of the receptor kinase activity and/or biological activity. This receptor domain displays a low degree of homology among the different receptor tyrosine kinases. Therefore, it is possible that this distal receptor domain contains specialized regions for interaction with cellular substrates and could, as such, play a crucial role in signal definition and receptor specificity. In such a model, receptor activation and/or phosphorylation of the C-terminal domain could change the conformation in this receptor part, leading to an improved interaction with cellular components. In the present work, we have approached the question of the role of the insulin receptor C-terminus in signal transmission by using antipeptides against two defined regions of this domain. The antibodies to peptide C1 (1309–1326) and to peptide C2 (1294–1317), called anti-C1 and anti-C2, respectively, recognized the human insulin receptor and reacted with distinct sites on the C-terminal domain. Our key observation was that these antipeptides were able to detect modifications in the receptor molecule, induced by hormone binding and by the phosphorylation state. Thus, compared to a monoclonal antibody (B6) known to precipitate the total amount of insulin receptor present, anti-C1 immunoprecipitated as much native receptor as antibody B6, but it was poorly efficient to precipitate the phosphorylated receptor or the insulin–receptor complex. Anti-C2 recognized the phosphoreceptor as well as antibody B6, but precipitated less receptor occupied by insulin or native receptor than antibody B6. As a whole, these data suggested to us that hormone binding and phosphorylation might induce conformational changes in the insulin receptor molecule. To investigate this possibility, we worked out a novel radioimmunoassay of the insulin receptor using ^{35}S-receptors. Radioimmunoassay of receptors using ^{125}I-insulin-labeled receptors has been previously described by Harrison et al. (1979) but could evidently not be applied in our study designed to detect hormone-induced conformational changes.

Our results with the ^{35}S-receptor radioimmunoassay show that receptor antibodies can detect two types of modifications in the insulin receptor, phosphorylation-induced ones and ligand-induced ones. Concerning the phosphorylation-induced changes, anti-C2 immunoprecipitated all the phosphoreceptors but not all the native receptors. This result can be compared to those obtained with antipeptides against the receptor tyrosine residues 1150–1151 (Herrera & Rosen, 1986; Perlman et al., 1989), indicating that conformational changes could be induced by phosphorylation. On the contrary, anti-C1 failed to recognize a fraction of the phosphoreceptor, indicating that receptor phosphorylation has resulted in the disappearance of certain epitopes. Further, receptor phosphorylation induced a decrease in the affinity of anti-C1 for the receptor compared to the native receptor. These results could be explained either by a phosphorylation-induced conformational change and/or by steric hindrance of anti-C1 recognition as a result of phosphorylation on tyrosine residues 1316 and/or 1322, which are probably part of the epitope recognized by the antipeptide. The latter hypothesis is supported by the fact that the phos-

phorylation-induced change in anti-C1 recognition persists after protein denaturation. Although other studies with antipeptides to the receptor C-terminus did not detect any conformational changes in this domain (Herrera et al., 1985; Grunfeld et al., 1987; Perlman et al., 1989), we cannot exclude the possibility of a phosphorylation-induced conformational change detected by anti-C2, but not by anti-C1, due to steric hindrance.

Our ^{35}S-receptor immunoassay was able to detect another type of change in the insulin–receptor molecule, which is provoked by ligand binding. When anti-C1 and anti-C2 antibodies were allowed to react with native receptors or with insulin–receptor complexes, pronounced changes in the total immunoprecipitation capacity were found. Only a reduced fraction of insulin–receptor complexes were precipitated by anti-C1 and anti-C2 compared to the total precipitation obtained with the native receptors. Moreover, the antipeptides had a slightly better affinity for liganded receptors than for native receptors. These data indicate that the interaction of insulin with the receptor has the same consequences on the domains recognized by the two different antibodies. Further, for both antipeptides, the insulin-induced change is not retained after denaturation of the receptor molecule, which indicates that the change in antipeptide recognition is reversible. The simultaneous occurrence of a loss of epitopes and a change in affinity for antibodies leads us to propose the following working hypothesis. The binding of insulin to its receptor results in a conformational change with at least two concomitant consequences: one resulting in a better antibody fit or improved recognition of epitope and another one leading to the masking of epitopes with disappearance of antibody recognition.

A striking feature of the conformational change induced by insulin in the receptor C-terminus is that it is independent of receptor phosphorylation and/or kinase activity, since insulin alone in the absence of ATP could induce the observed phenomenon. Note that for other insulin receptor domains ligand-induced phosphorylation has been shown to produce conformational changes (Herrera & Rosen, 1986; Perlman et al., 1989). Similarly, phosphorylation of the PDGF–receptor results in conformational changes (Bishayee et al., 1988; Keating at al., 1988).

Evidence that structural changes occur at the level of the α-subunit upon insulin binding (Pilch & Czech, 1980; Donner & Yonkers, 1983; Waugh & Pilch, 1989) and at the level of the β-subunit upon autophosphorylation (Herrera & Rosen, 1986; Perlman et al., 1989) has led to the concept that insulin binding to the α-subunit results in conformational changes leading to signal transmission and receptor kinase activation. Our present work lends added weight to this proposal and provides original evidence that these changes are transmitted to the receptor β-subunit C-terminal portion. These changes in the receptor cytoplasmic domain could influence interactions between the receptor and cellular structures and, as such, play a key role in signal transduction.

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Effects of General Anesthetics on the Bacterial Luciferase Enzyme from *Vibrio harveyi*: An Anesthetic Target Site with Differential Sensitivity†

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ABSTRACT: The effects of a diverse range of 36 general anesthetics and anesthetic-like compounds on a highly purified preparation of the bacterial luciferase enzyme from *Vibrio harveyi* have been investigated. Under conditions where the flavin site was saturated, almost all of the anesthetics inhibited the peak enzyme activity and slowed the rate of decay. However, a small number of the more polar agents only inhibited at high concentrations, while stimulating activity at lower concentrations. The inhibition was found to be competitive in nature, with the anesthetics acting by competing for the binding of the aldehyde substrate *n*-decanal. The anesthetic binding site on the enzyme could accommodate only a single molecule of a large anesthetic but more than one molecule of a small anesthetic, consistent with the site having circumscribed dimensions. The homologous series of *n*-alcohols and *n*-alkanes exhibited cutoffs in inhibitory potency, but these cutoffs occurred at very different chain lengths (about C_{10} for the *n*-alkanes and C_{15} for the *n*-alcohols), mimicking similar cutoffs observed for general anesthetic potencies in animals. Binding constants determined from peak height measurements showed that the inhibitor binding site was predominantly hydrophobic (with a mean $\Delta\Delta G^{\ominus}_{CH_2}$ of −5.0 kJ/mol), but fluctuations in the binding constants with chain length revealed regions in the binding site with polar characteristics. Binding constants to an intermediate form of the enzyme (intermediate II) were also determined, and these confirmed the principal features of the binding site deduced from the peak height measurements. The long-chain compounds, however, bound considerably tighter to the intermediate II form of the enzyme, and this was shown to account for the biphasic decay kinetics that were observed with these compounds. Overall, there was poor agreement between the EC_{50} concentrations for inhibiting the luciferase enzyme from *V. harveyi* and those which induce general anesthesia in animals, with bulky compounds being much less potent, and moderately long chain alcohols being much more potent, as luciferase inhibitors than as general anesthetics.

Many years ago it was noted that the presence of relatively low levels of general anesthetics inhibited the light emitted by cultures of various bioluminescent bacteria (Harvey, 1915; Taylor, 1934; Johnson et al., 1951). Subsequently, it was shown that a good correlation existed between the concentrations required to reduce light output from these bacteria and those required to induce general anesthesia in animals (Halsey & Smith, 1970; White & Dundas, 1970). The possible importance of these findings for understanding the molecular mechanisms that underlie general anesthesia is obvious and further enhanced when it is appreciated that the remarkable observation of the pressure reversal of general anesthesia in animals (Johnson & Flagler, 1950) was stimulated by the observation of similar effects in bioluminescent bacteria (Johnson et al., 1942). It is all the more surprising, therefore, that studies of the effects of general anesthetics on the bacterial luciferase enzyme itself have been so few and far between, despite considerable advances in understanding the kinetics and catalytic mechanism of the uninhibited enzyme [see Ziegler and Baldwin (1981) and Hastings et al. (1985) for reviews].

The few studies that have been reported over the last 2 decades have employed relatively impure preparations of the enzyme and have used only a handful of anesthetic agents (White et al., 1973; Adey et al., 1976; Middleton & Smith, 1976a,b; Banks & Peace, 1985). An added confusion is that different species of bacteria have been used in the various studies, and it is known that, at least in some cases, there can be marked species variations [see, for example, Hastings et al. (1969)]. While these studies have shown clearly that the bacterial luciferase enzyme is sensitive to at least some general anesthetics and that the predominant mode of inhibition is competitive in nature, the impression that is often given is that all luciferase enzymes are very sensitive to general anesthetics and, moreover, that the anesthetic response of the bacterium derives almost entirely from the effects of anesthetics on the

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