The Insulin Receptor C-Terminus Is Involved in Regulation of the Receptor Kinase Activity†

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ABSTRACT: During the insulin receptor activation process, ligand binding and autophosphorylation induce two distinct conformational changes in the C-terminal domain of the receptor β-subunit. To analyze the role of this domain and the involvement of the C-terminal autophosphorylation sites (Tyr1316 and Tyr1322) in receptor activation, we used (i) antipeptide antibodies against three different C-terminal sequences (1270–1281, 1294–1317, and 1309–1326) and (ii) an insulin receptor mutant (Y/F2) where Tyr1316 and Tyr1322 have been replaced by Phe. We show that the autophosphorylation-induced C-terminal conformational change is preserved in the Y/F2 receptor, indicating that this change is not induced by phosphorylation of the C-terminal sites but most likely by phosphorylation of the major sites in the kinase domain (Tyr1146, Tyr1150, and Tyr1151). Binding of antipeptide antibodies to the C-terminal domain modulated (activated or inhibited) both mutant and wild-type receptor-mediated phosphorylation of poly(Glu/Tyr). In contrast to the wild-type receptor, Y/F2 exhibited the same C-terminal configuration before and after insulin binding, evidencing that mutation of Tyr1316 and Tyr1322 introduced conformational changes in the C-terminus. Finally, the mutant receptor was 2-fold more active than the wild-type receptor for poly(Glu/Tyr) phosphorylation. In conclusion, the whole C-terminal region of the insulin receptor β-subunit is likely to exert a regulatory influence on the receptor kinase activity. Perturbations of the C-terminal region, such as binding of antipeptides or mutation of Tyr1316 and Tyr1322, provoke alterations at the receptor kinase level, leading to activation or inhibition of the enzymic activity.

The insulin receptor is a transmembrane glycoprotein which contains a cytoplasmic tyrosine kinase activated by ligand binding to the extracellular α-subunit (Ullrich et al., 1985; Ebina et al., 1985). Activation of the kinase involves multisite autophosphorylation of the receptor on tyrosine residues (Kasuga et al., 1982; Van Obberghen & Kowalski, 1982; Van Obberghen et al., 1983). The major autophosphorylation sites of the insulin receptor are clustered in two domains. The first one is found in the tyrosine kinase domain and includes tyrosine residues 1146, 1150, and 1151, which are homologous to the major in vitro autophosphorylation sites of $pp60^{v\text{-}src}$ (Tornqvist et al., 1987). The autophosphorylation of this domain is closely linked to full activation of the insulin receptor kinase (Ellis et al., 1986; Wilden et al., 1992).

The second autophosphorylation domain, located in the C-terminal region, contains tyrosine residues 1316 and 1322. During the insulin receptor activation process, this domain undergoes two distinct conformational changes: the first upon insulin binding and the second following autophosphorylation (Baron et al., 1992). However, the involvement of this domain in the receptor biological functioning remains controversial. Indeed, in rat 1 fibroblasts, a truncated insulin receptor lacking the last 43 amino acids of the β-subunit, which include Tyr1316 and Tyr1322 (HIRΔ43), has been shown to be a poor mediator of the receptor's metabolic effects but an active growth signaler compared to the wild-type receptor (Maegawa et al., 1988; Scott-Thies et al., 1989). In contrast to these reports, when the truncated receptor was transfected in CHO cells, the activity of the truncated receptor was found to be identical to that of the intact receptor (Myers et al., 1991). Moreover, the expression of a mutated insulin receptor, where Tyr1316 and Tyr1322 are replaced by Phe (Y/F2), in either rat 1 fibroblasts (Takata et al., 1991, 1992) or CHO cells (Ando et al., 1992) leads to a normal transmission of insulin-stimulated metabolic signals, while the sensitivity to insulin-stimulated DNA synthesis is markedly enhanced.

To define more precisely the function of the insulin receptor C-terminal domain, we studied both Y/F2 mutant and wild-type insulin receptors using antipeptide antibodies to the following consecutive sequences of the β-subunit C-terminal domain: (i) 1270–1281, (ii) 1294–1317, containing Tyr1316, and (iii) 1309–1326, containing Tyr1316 and 1322. We provide evidence for the existence of bidirectional interactions between the kinase domain and the C-terminal domain through conformational changes. Indeed, autophosphorylation of the major sites in the kinase domain (Tyr1146, Tyr1150, and Tyr1151) induces a C-terminal conformational change. Likewise, perturbations of the C-terminal region, such as mutation of Tyr1316 and 1322 or binding of antipeptides, provoke alterations at the receptor kinase level reflected by activation or inhibition of the receptor kinase. Thus, the whole C-terminus appears to be involved in regulation of the receptor kinase activity.

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MATERIALS AND METHODS

Insulin Receptor Partial Purification. Rat 1 fibroblasts, transfected with expression plasmids encoding the wild-type human insulin receptor lacking the 12 amino acids corresponding to exon 11, were a gift from Dr. A. Ullrich, Max-Planck Institute für Biochimie, Munich, Germany. Rat 1 fibroblasts expressing the mutant insulin receptor Y/F2 (tyrosine residues 1316 and 1322 mutated to Phe) were produced as previously described (Takata et al., 1991, 1992).

Wild-type and Y/F2 insulin receptors were partially purified by chromatography on wheat germ agglutinin (WGA) as previously reported (Van Obberghen et al., 1981). Briefly, cells were solubilized for 90 min at 4 °C in 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, and 10% glycerol, pH 7.6, supplemented with 1 mM EGTA and 1.5 mM $MgCl_2$. The supernate from an ultracentrifugation step (60 min, 100 000*g*, 4 °C) was applied to a WGA column, and receptors were eluted with 3 M *N*-acetyl-D-glucosamine in 50 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, pH 7.6. Protease inhibitors were present throughout the procedure (2 μM leupeptin, 1.25 mM bacitracin, 100 units/mL aprotinin, and 1 mM phenylmethanesulfonyl fluoride).

Receptors biosynthetically labeled with [^{35}S]methionine were obtained as previously described (Van Obberghen et al., 1981).

Production of Antipeptide Antibodies. Antipeptide antibodies to four insulin proreceptor sequences were produced: (i) the extracellular sequence 456–465 of the receptor α-subunit and (ii) three consecutive C-terminal sequences in the receptor β-subunit, (a) 1270–1281, (b) 1294–1317, containing the Tyr1316, one of the residues mutated in Y/F2 receptor, and (c) 1309–1326, including the two tyrosine residues mutated in Y/F2 receptor, Tyr1316 and Tyr 1322. In this study, we used the numbering system published by Ullrich et al. (1985).

The peptides were coupled to keyhole limpet hemocyanin and injected intradermally into rabbits as described earlier (Yoshitake et al., 1982). Antipeptides detected by enzyme-linked immunosorbent assay were partially purified by chromatography on protein A–sepharose. Elution was performed with 1 M glycine and 0.5 M NaCl, pH 2, and immediate neutralization with 1 M Tris, pH 8. Immunoglobulins were dialyzed against 20 mM HEPES, 60 mM NaCl, pH 7.5.

Immunoblotting. Wild-type and Y/F2 insulin receptors were treated with 10^{-7} M insulin for 1 h at 22 °C. Identical amounts of activated receptor were then incubated with 8 mM $MgCl_2$ and 4 mM $MnCl_2$ in the presence or absence of 50 μM ATP for 1 h at 22 °C. Laemmli sample buffer containing 3% SDS and 5% β-mercaptoethanol was then added, and samples were analyzed by one dimensional SDS/polyacrylamide gel electrophoresis (Laemmli, 1970). Western blotting was performed on Immobilon-P membranes (Millipore, Bedford, MA) as described by Towbin et al. (1979), using defatted skimmed milk 5% (w/v) to block nonspecific binding. The membranes were incubated with rabbit immune sera (1/100) overnight at 4 °C and then extensively washed with 10 mM Tris buffers containing 0.5 M NaCl and 0.1%, 0.2%, or 0.5% Tween, alternatively. After incubation with [^{125}I]protein A and washing as described above, the membranes were dried and submitted to autoradiography. Nonimmune serum was used as a control.

Substrate Phosphorylation. (A) Effect of C-Terminal Antipeptide Antibodies on Y/F2 Kinase Activity. Y/F2 receptor tyrosine kinase activity toward the exogenous substrate poly(Glu/Tyr) was measured after incubation of solubilized receptor with the partially purified antipeptide antibodies for 2 h at 4 °C. Receptors were then treated or not with 10^{-7} M insulin for 60 min at room temperature, and phosphorylation of poly(Glu/Tyr) (0.2 mg/mL or the indicated concentrations) was carried out for 45 min at the same temperature in 8 mM $MgCl_2$, 4 mM $MnCl_2$, and 15 μM [γ-^{32}P]ATP (2.5 Ci/mmol). Incorporation of radioactive phosphate into the substrate was measured as previously described using a filter paper assay (Braun et al., 1984; Le Marchand-Brustel et al., 1985).

(B) Poly(Glu/Tyr) and ATP Dependence of Wild-Type and Y/F2 Receptors. Identical amounts of wild-type and Y/F2 receptors (determined by ^{125}I-insulin binding) were incubated in the presence or absence of 10^{-7} M insulin for 1 h at 22 °C. We compared the wild-type and Y/F2 receptors' affinity for poly(Glu/Tyr) by varying the substrate concentration from 0.1 to 2 mg/mL under the phosphorylation conditions described above. The ATP concentration dependence of wild-type and Y/F2 receptor kinase activity was analyzed by phosphorylating poly(Glu/Tyr) (2 mg/mL) at increasing ATP concentrations ranging from 7.5 to 60 μM. Under the assay conditions, the reaction was verified to be linear for at least 90 min (data not shown).

Immunoprecipitation of ^{35}S-Labeled Unoccupied or Insulin-Bound Receptors. Increasing amounts of ^{35}S-labeled receptors (ranging from 0.15 to 2.4 pmol as determined by Scatchard analysis) were incubated or not with 10^{-7} M insulin overnight at 4 °C. Antipeptide antibodies to insulin receptor sequence 1309–1326 were then added at a constant concentration (50 μg/mL) for 2 h at 4°C in a final volume of 50 μl. Protein A–sepharose was used to immunoprecipitate the complex and immune pellets were washed three times with 50 mM HEPES and 150 mM NaCl containing 0.1% Triton X-100. Pellets were resuspended in 1 mL of scintillation solution for counting.

RESULTS

Role of Tyr1316 and Tyr1322 in Autophosphorylation-Induced C-Terminal Conformational Changes. We have shown that the insulin receptor cytoplasmic domain undergoes conformational changes after autophosphorylation (Baron et al., 1992). One of them is detected in the C-terminal region, where two of the receptor autophosphorylation sites are clustered (Tyr1316 and Tyr1322). To determine whether this change is dependent on the two C-terminal tyrosines, we used an insulin receptor mutant with these two residues replaced by phenylalanine (Y/F2 receptor). Unphosphorylated or phosphorylated wild-type and Y/F2 receptors were immunoblotted with an antipeptide directed to the receptor sequence (1309–1326) (Figure 1, left panel). To rule out degradation of the β-subunit, we performed a similar immunoblot experiment with another C-terminal antibody, anti-(1294–1319) (Figure 1, right panel). We have previously shown that in western blotting experiments this antipeptide does not differentiate between unphosphorylated and phosphorylated wild-type insulin receptors (Baron et al., 1990). The value obtained with the unphosphorylated receptor blotted with anti-(1309–1326) was chosen as 100% to allow comparison of the different experiments. Using scanning of four independent experiments, with the wild-type receptors, we found that anti-(1309–1326) blotted the phosphorylated receptors at 52 ± 6%, whereas anti-(1294–1317) blotted 61 ± 9 and 69 ± 32% of unphosphorylated and phosphorylated receptors, respectively.

Concerning the Y/F2 receptor, anti-(1309–1326) blotted 36 ± 14% of the phosphoreceptors, and anti-(1294–1317)

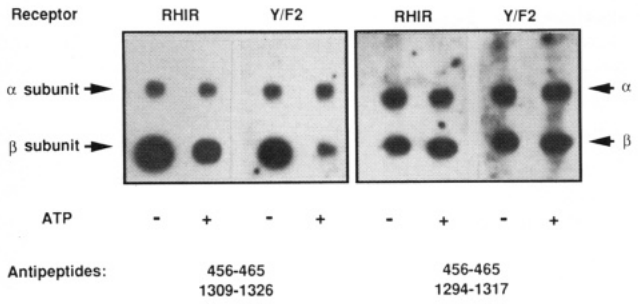

FIGURE 1: Immunoblotting of the unphosphorylated and phosphorylated forms of wild-type and Y/F2 receptors. Wild-type and Y/F2 receptors were activated with 10^{-7} M insulin for 1 h at 22 °C. Identical amounts of these activated receptors were treated with 8 mM $MgCl_2$ and 4 mM $MnCl_2$, in the absence or in the presence of 50 μM ATP for 1 h at 22 °C. The samples were then submitted to SDS-PAGE under reducing conditions. After electrophoresis, the proteins were transferred to nitrocellulose, and the cellulose membrane was incubated overnight at 4 °C with a mixture of antipeptide antibody to the insulin receptor α-subunit, anti-(456–465), together with either anti-(1309–1326) (left panel) or anti-(1294–1317) (right panel), both directed against the insulin receptor β-subunit. [^{125}I]protein A was added for 1 h at room temperature. The nitrocellulose membrane was dried and subjected to autoradiography. Immunosera were used at a 1/100 dilution. Sera obtained from noninjected rabbits were used as controls (data not shown). The figure is representative of four independent experiments.

blotted 60 ± 21 and 42 ± 2% of unphosphorylated and phosphoreceptors, respectively. Thus, the antipeptide directed to the sequence containing the two tyrosine residues (1309–1326), recognized the β-subunit of the phosphorylated receptors to a much lesser extent than the β-subunit of the unphosphorylated receptors. By contrast, the other antipeptide, anti-(1294–1317), did not clearly discriminate between the two receptor forms.

The specificity of the domain recognized by the antipeptide antibody was verified by immunoblotting the receptors in the absence or in the presence of the synthetic insulin receptor peptide 1309–1326. At a concentration of 10^{-5} M, the peptide completely abolished the interaction of the antipeptide with the receptor β-subunit (data not shown). To demonstrate that the amount of receptor subjected to immunoblotting was the same in both conditions (phosphorylated or not), we included an antipeptide antibody which recognizes the α-subunit, anti-(456–465) (Figure 1). To conclude, the results shown in Figure 1 indicate that after autophosphorylation there is an alteration in the carboxyl terminus of the β-subunit, with the disappearance of epitopes recognized by anti-(1309–1326). This C-terminal modification is preserved in the Y/F2 receptor, which lacks the two C-terminal autophosphorylation sites. Therefore, neither the presence of Tyr1316 and Tyr1322 nor their autophosphorylation is involved in the C-terminal change accompanying the autophosphorylation of the insulin receptor. Moreover, the C-terminal conformation of the phosphorylated receptor is such that the receptor refolds when removed from denaturants and immobilized on the membrane for the immunoblotting assay.

Modulation of Insulin Receptor Kinase Activity by Antipeptide Antibodies to β-Subunit C-Terminal Domains. We have previously reported that the antipeptide antibody to the insulin receptor sequence 1309–1326, which includes Tyr1316 and Tyr1322, is an activator of the receptor kinase, both in vitro and in vivo (Baron et al., 1991). To analyze if the antibody effect was dependent on the two C-terminal autophosphorylation sites, poly(Glu/Tyr) phosphorylation was measured after incubation of Y/F2 receptors at increasing anti-(1309–1326) concentrations. As seen in Figure 2, this

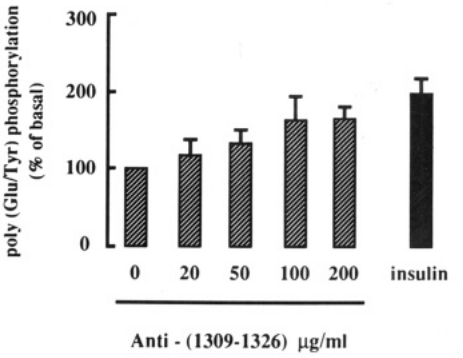

FIGURE 2: Effect of anti-(1309–1326) on Y/F2 receptor kinase activity. Partially purified Y/F2 receptors were incubated at increasing concentrations of anti-(1309–1326) or nonimmune Ig for 2 h at 4 °C prior to addition of buffer or insulin (10^{-7} M) for 1 h at 22 °C. Phosphorylation of poly(Glu/Tyr) (0.2 mg/mL) was then initiated as described under Materials and Methods. After 45 min at 22 °C, the reaction was stopped, and samples were analyzed using a filter paper assay. The mean ± SEM of three independent experiments, where each point was run in triplicate, is shown.

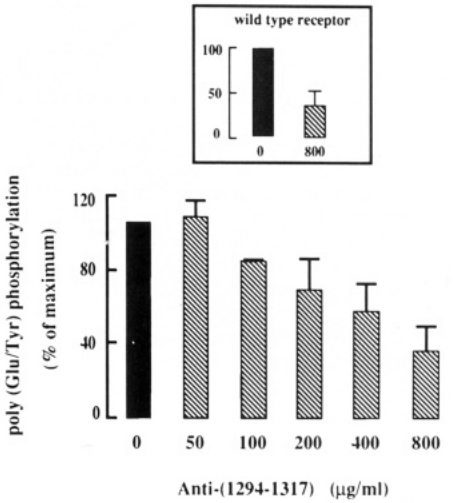

FIGURE 3: Effect of anti-(1294–1317) on Y/F2 and wild-type insulin receptor kinase activity. Partially purified receptors were incubated with increasing concentrations of anti-(1294–1317) (hatched bars) or nonimmune Ig (closed bars) for 2 h at 4 °C, prior to addition of insulin (10^{-7} M) for 1 h at 22 °C. Phosphorylation of poly(Glu/Tyr) (0.2 mg/mL) was then initiated as described under Materials and Methods. After 45 min at 22 °C, the reaction was stopped, and samples were analyzed using a filter paper assay. Inset: Wild-type insulin receptors were incubated with nonimmune Ig or anti-(1294–1317) (800 μg/mL) in the presence of 10^{-7} M insulin, and then phosphorylation of poly(Glu/Tyr) (0.2 mg/mL) was carried out as described above. The mean ± SEM of three independent experiments, where each point was run in triplicate, is shown.

antipeptide antibody enhanced Y/F2 receptor kinase activity in a dose-dependent manner in the absence of insulin. Similar to the wild-type receptor, anti-(1309–1326) was without detectable effect in the presence of insulin (data not shown).

Next we wanted to see if the ability to modulate the receptor kinase activity was restricted to the sequence including the two C-terminal phosphorylation sites. To this end, we tested the effect of antipeptide antibodies directed against two other C-terminal sequences, 1294–1317 and 1270–1281. As shown in Figure 3, anti-(1294–1317) exerted a dose-dependent inhibition on insulin-stimulated phosphorylation of poly(Glu/Tyr) by the Y/F2 receptor. Similar effects were obtained for the wild-type receptor. For both receptors, this inhibition was also observed in the absence of insulin (data not shown). Finally, anti-(1270–1281) was found to decrease insulin-stimulated Y/F2 receptor kinase activity by 60% at poly(Glu/

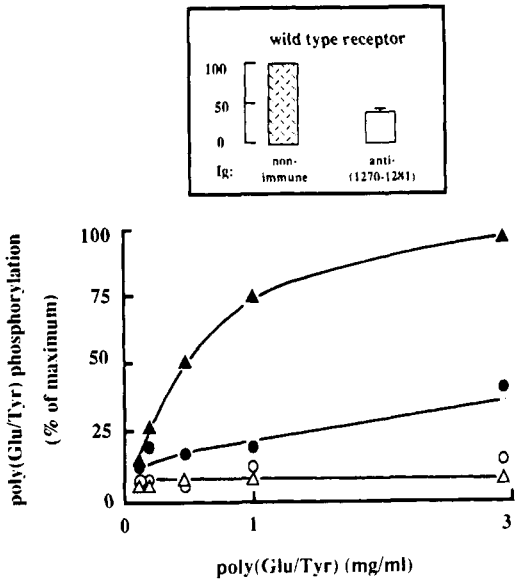

FIGURE 4: Effect of anti-(1270–1281) on phosphorylation of poly(Glu/Tyr) by Y/F2 and wild-type insulin receptors. Partially purified Y/F2 receptors were incubated with anti-(1270–1281) or nonimmune Ig (0.4 mg/mL) for 2 h at 4 °C prior to addition of buffer (open symbols) or 10^{-7} M insulin (closed symbols) for 1 h at 22 °C. Phosphorylation was then initiated as described under Materials and Methods at increasing concentrations of poly(Glu/Tyr). After 45 min at 22 °C, the reaction was stopped, and samples were analyzed using a filter paper assay. The mean of two independent experiments, where each point was run in triplicate, is shown. (○, ●) anti-(1270–1281); (△, ▲) nonimmune Ig. Inset: Wild-type insulin receptors were incubated with anti-(1270–1281) or nonimmune Ig (0.4 mg/mL) in the presence of 10^{-7} M insulin, and then phosphorylation of poly(Glu/Tyr) (1 mg/mL) was carried out as described above. The mean ± SEM of three independent experiments, where each point was run in triplicate, is shown.

Tyr) concentrations ranging from 0.2 to 3 mg/mL and did not affect basal phosphorylation (Figure 4). A similar effect was observed for the wild-type receptor.

As a whole, these data allow us to conclude that, for both Y/F2 and wild-type receptors, the C-terminal region is likely to exert a regulatory role on the receptor kinase activity.

Insulin-Induced Carboxyl Terminus Conformational Changes. The Y/F2 receptor exhibits unaltered ligand binding and capacity to transmit metabolic effects. In contrast, it has a growth-promoting action which is characterized by an enhanced insulin sensitivity (Takata et al., 1991, 1992; Ando et al., 1992). In a previous study, we have documented a C-terminal conformational change in the cytoplasmic β-subunit after hormone binding to the extracellular α-subunit. This change was unrelated to ATP binding, as it was conserved in a kinase-deficient mutant receptor which does not bind ATP (Baron et al., 1992). We have proposed that this insulin-induced conformational modification leads to a preactive form of the receptor. Therefore, it was of interest to determine whether the Y/F2 receptor changed its conformation in response to hormone binding. To this end, ^{35}S-labeled receptors lectin-purified from cells incubated in absence of insulin were exposed or not to insulin, as described in Materials and Methods. These receptors were certainly unphosphorylated, since no ATP was added during the in vitro procedure. Increasing amounts of each pool of ^{35}S-labeled receptors were then subjected to precipitation with a constant concentration of anti-(1309–1326) (50 μg/mL). As seen in Figure 5, left panel, this antipeptide antibody was unable to distinguish between unoccupied and ligand-bound Y/F2 receptors. This result clearly differs from that obtained with the wild-type receptor, where the polyclonal antibody anti-(1309–1326) shows approximately a 40% decrease in its capacity to precipitate insulin-bound receptors compared to unoccupied ones (Figure 5, right panel).

Comparison of the ATP and Poly(Glu/Tyr) Concentration Dependency of the Y/F2 and Wild-Type Insulin Receptor Kinases. Two observations led us to hypothesize that the Y/F2 native conformation could correspond to a more active kinase. The first one is that mutation of Tyr1316 and Tyr1322 introduces a structural alteration in the Y/F2 receptor C-terminus as, in contrast to the wild-type receptor, the mutated receptor does not undergo an insulin-induced conformational change. The second observation is that, compared to the wild-type receptor, the Y/F2 receptor demonstrates enhanced insulin sensitivity in signaling certain biological effects. To test the idea of a more active kinase, we compared the ATP dependence of the Y/F2 receptor with that of the wild-type receptor. This was accomplished by measuring phosphorylation of the exogenous substrate poly(Glu/Tyr) by both receptors at increasing ATP concentrations, in the absence or in the presence of insulin. In Figure 6A, a double-reciprocal plot of the data showed that for the Y/F2 and wild-type receptors, affinity for ATP was the same. Moreover, the values for basal poly(Glu/Tyr) phosphorylation by the Y/F2 receptor were undistinguishable from those found with the insulin-stimulated wild-type receptor. Further, in the presence of insulin and at each ATP concentration tested, the Y/F2 receptor kinase activity was 2-fold higher compared to that of the wild-type receptor. To determine whether the Y/F2 receptor kinase augmentation in V_{max} was due to an increase in ATP exchange rate or to a higher receptor affinity for substrate, we compared the kinase activity of Y/F2 and wild-type receptors at increasing poly(Glu/Tyr) concentrations. As shown in Figure 6B, both receptors exhibited the same affinity for the substrate, whereas the V_{max} of the phosphorylation reaction was increased for Y/F2 receptors. We conclude from these data that replacement of the two C-terminal tyrosines by phenylalanines in the Y/F2 receptor renders the kinase more active both in presence and in the absence of insulin. This increase in activity is observed without detectable changes in affinity for ATP or for the synthetic substrate.

DISCUSSION

We have studied the involvement of the insulin receptor β-subunit C-terminus and, in particular, the role of the two C-terminal autophosphorylation sites (Tyr1316 and Tyr1322) in the receptor activation process. The molecular mechanism leading to insulin receptor activation involves several conformational changes following ligand binding, ATP binding, and autophosphorylation (Herrera & Rosen, 1986; Maddux & Goldfine, 1991; Baron et al., 1992). Conformational changes associated with autophosphorylation appear to be part of a general mechanism in the activation process of tyrosine kinase receptors. Indeed, the receptors for platelet-derived growth factor (Keating et al., 1988; Bishayee et al., 1988) and for colony-stimulating factor-1 (Downing et al., 1991) also undergo conformational changes which are strictly dependent on receptor kinase activity and/or autophosphorylation. In the present study on insulin receptors, we found that, similar to the wild-type receptor, after autophosphorylation the C-terminal domain of Y/F2 receptor adopts a form clearly distinguishable from the native one. Due to the absence of Tyr1316 and Tyr1322 in the Y/F2 receptor, the C-terminal alteration is obviously independent of the C-ter-

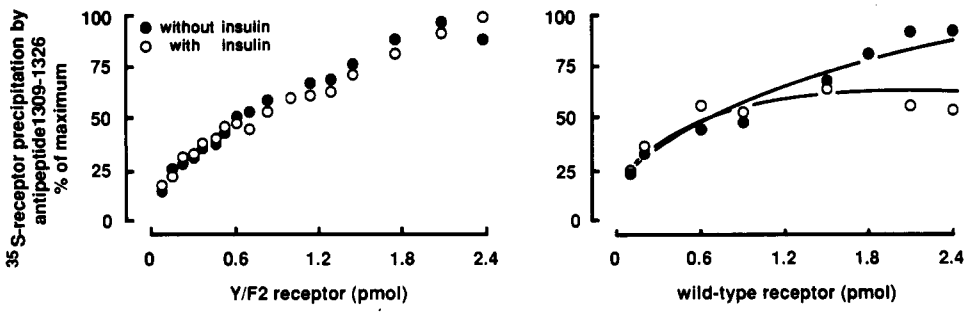

FIGURE 5: Anti-(1309–1326) precipitation of ^{35}S-labeled Y/F2 and wild-type receptors. ^{35}S-labeled Y/F2 and wild-type receptors were incubated overnight at 4 °C in the absence (●) or in the presence (○) of 10^{-7} M insulin. Increasing concentrations of these ^{35}S-receptors were then added to anti-(1309–1326) (50 μg/mL) for 2 h at 4 °C. After incubation with protein A, the pellets were washed and resuspended as described in Materials and Methods. The data shown are the mean of two independent experiments, where each point was run in duplicate. The nonspecific background was determined in the presence of nonimmune Ig.

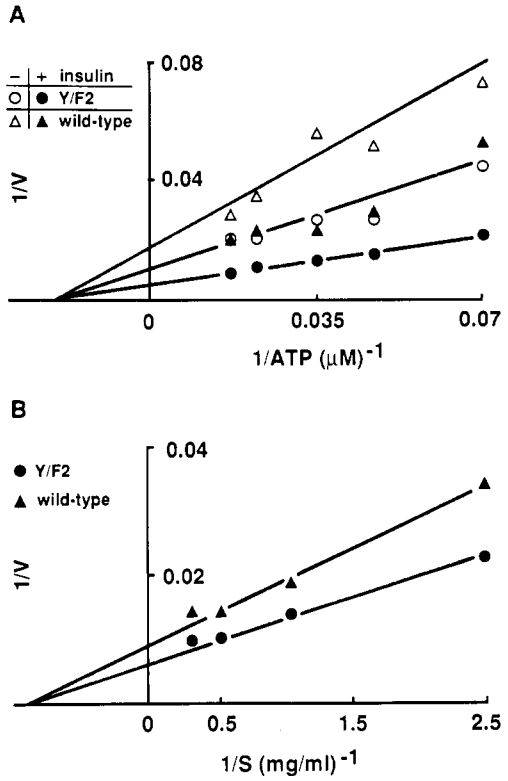

FIGURE 6: Analysis of Y/F2 and wild-type receptor kinase in the presence of varying concentrations of ATP or poly(Glu/tyr). Identical amounts of Y/F2 and wild-type insulin receptors were incubated with 10^{-7} M insulin (closed symbols) or with buffer (open symbols) for 1 h at 22 °C. Panel A: phosphorylation of the synthetic substrate poly(Glu/Tyr) (2 mg/mL) was initiated under the conditions described in Materials and Methods at increasing concentrations of [γ-^{32}P]ATP ranging from 7.5 to 60 μM. After 45 min at 22 °C, the reaction was stopped, and samples were analyzed using a filter paper assay. The mean of three independent experiments, where each point was run in triplicate, is presented according to Lineweaver–Burk. Panel B: Phosphorylation was performed in the presence of increasing concentrations of poly(Glu/Tyr) ranging from 0.1 to 3 mg/mL. $1/V$ represents the inverse of the percentage of the maximal effect (phosphorylation by Y/F2 receptor at the highest concentration of poly(Glu/Tyr)). The means of triplicates obtained from a representative experiment are analyzed according to a Lineweaver–Burk plot.

minus autophosphorylation sites. Hence, the conformational change is induced very likely by autophosphorylation of the major sites in the kinase domain (Tyr1146, Tyr1150, and Tyr1151). Autophosphorylation of these kinase domain tyrosines has been shown to be essential for the enzymic activity of the insulin receptor (Ellis et al., 1986; Wilden et al., 1992). The fact that the C-terminal conformational change appears to be induced by phosphorylation of these sites (Tyr1146, Tyr1150, and Tyr1151) further illustrates that it is an important event in receptor activation.

Previous reports showed that the Y/F2 receptor expressed in rat 1 fibroblasts or in CHO cells (Takata et al., 1991, 1992; Ando et al., 1992) exhibits an enhanced insulin sensitivity to transmit mitogenic signals, while insulin's stimulatory action on glucose transport is unchanged. Our present work provides a plausible molecular explanation for these observations, as we found at least two modifications in the Y/F2 receptor, i.e., (i) an increased kinase activity and (ii) an altered configuration. We would like to suggest that the conjunction of both modifications is responsible—at least in part—for the increased insulin sensitivity for the mitogenic response. According to this view, the altered receptor configuration would favor interactions solely with molecules involved in growth-promoting effects. This situation is reminiscent of our previous work, where we found that microinjection of an antipeptide to the C-terminus sequence 1309–1326 stimulates growth but not glucose nor amino acid uptake (Baron et al., 1991). Concerning the Y/F2 receptor kinase, we found that it is 2-fold more active than the wild-type receptor when poly(Glu/Tyr) is used as an in vitro substrate. In trying to explain the enhanced activity of the Y/F2 compared to the wild-type receptor, we looked at their C-terminal conformations before and after insulin binding. Indeed, it has been shown that during activation of the wild-type insulin receptor, there is a C-terminal conformational change induced by insulin prior to receptor autophosphorylation (Baron et al., 1992). Further, in a recent study we observed that the insulin-induced C-terminal conformational change correlates with the hormone's ability to stimulate receptor autophosphorylation and, therefore, might be essential for signal transmission from the extracellular to the intracellular domains of the receptor (Lebrun et al., 1993). Here we report that a hormone-induced C-terminal conformational change does not take place in the Y/F2 receptor, which exhibits the same configuration before and after insulin binding. This result, together with the facts that in vitro Y/F2 kinase is more active than the wild-type kinase and that Y/F2 cells are more sensitive to insulin for certain biological effects, lead us to propose that the insulin-insensitive C-terminal conformation of the Y/F2 receptor corresponds to an active insulin receptor form. Interestingly, even though the Y/F2 kinase is more active than the wild-type, it remains stimulatable by insulin. This suggests that insulin binding and the mutations of Tyr1316 and Tyr1322 regulate the kinase via distinct mechanisms. Consistent with our results, Takata et al. (1992) have recently shown that the phosphotyrosine content of two insulin receptor substrates (pp180 and pp220) was greater in Y/F2 cells than in wild-

type cells, especially in the basal state. However, insulin sensitivity for phosphorylation of both substrates was the same in Y/F2 and wild-type cell lines. We interpret these data to mean that mutation of the two C-terminal tyrosines does not mimic the stimulation by insulin but rather affects a sequence which is involved in modulation of the in vitro and in vivo receptor kinase activity and which favors interaction involved in transduction of the growth promoting action of insulin. Consistent with this, the kinase activity of wild-type and Y/F2 receptors can be altered by antipeptide antibodies directed against C-terminal sequences spanning residues 1270 to 1326. The modulatory effects exerted by our antibodies are either inhibitory [anti-(1270–1281) and anti-(1294–1317)] or stimulatory [anti-(1309–1326)].

We conclude from our data that the whole C-terminus of the insulin receptor β-subunit is likely to exert a regulatory influence on the receptor kinase activity. Moreover, a bidirectional interplay based on conformational changes appears to occur between the kinase domain and the C-terminal domain. Indeed, receptor activation involves as an initial step phosphorylation of the three kinase domain tyrosine sites. This event induces a C-terminal conformational change which is independent of the phosphorylation of the C-terminal sites. Reciprocally, perturbations of the C-terminal region, such as the mutation of Tyr1316 and Tyr1322 and the binding of antipeptides, provoke alterations in the receptor kinase with resulting activation or inhibition of the enzymic activity.

Using the C-terminal truncated insulin receptor Δ43, Myers et al. (1991) have shown that the C-terminus plays a minimal role in insulin receptor functioning and signal transmission in CHO cells. In contrast, other groups have proposed that the C-terminal domain could function as a point of divergence between the metabolic and the mitogenic pathways of signal transmission by the insulin receptor (Scott-Thies et al., 1989; Baron et al., 1991; Takata et al., 1991, 1992; Ando et al., 1992). Our results indicate that the kinase domain of the insulin receptor is sensitive to stimuli coming from the C-terminal domain. It would be of interest to clarify whether kinase regulation by the C-terminus is involved in defining specificity of the biological signals elicited by the insulin receptor. How a receptor kinase modulation by the C-terminal domain occurs in vivo remains to be elucidated. It is tempting to speculate that after insulin receptor activation the C-terminal conformational change induced by the phosphorylation of Tyr1146, Tyr1150, and Tyr1151 in the kinase domain could favor regulatory molecules to bind to or to dissociate from the receptor C-terminus. Such a phenomenon could, in turn, modulate the receptor kinase activity. This model could explain that the binding of antibodies to C-terminal sequences and the mutation of Tyr1316 and Tyr1322 alter the insulin receptor kinase by perturbing the tertiary structure of this C-terminal regulatory region.

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