# The insulin receptor with phenylalanine replacing tyrosine-1146 provides evidence for separate signals regulating cellular metabolism and growth

PETER A. WILDEN, JONATHAN M. BACKER, C. RONALD KAHN, DEBORAH A. CAHILL, GREGORY J. SCHROEDER, AND MORRIS F. WHITE

Research Division, Joslin Diabetes Center, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02215

ABSTRACT    We have studied the function of a mutant insulin receptor (IR) molecule in which Tyr-1146, one of the first autophosphorylation sites in the $\beta$ subunit, was replaced with phenylalanine ($IR_{F1146}$). Autophosphorylation of the partially purified $IR_{F1146}$ was reduced 60–70% when compared to the wild-type IR but was still stimulated by insulin. The phosphotransferase activity of the dephospho form of both the IR and $IR_{F1146}$ toward exogenous substrates was stimulated 3- to 4-fold by insulin. However, the wild-type IR was activated 12-fold by autophosphorylation, whereas the $IR_{F1146}$ was activated only 2-fold. When the $IR_{F1146}$ was expressed in Chinese hamster ovary (CHO) cells, insulin binding was normal, whereas autophosphorylation was reduced 80% when compared to cells expressing the wild-type IR. Endogenous substrates of the insulin receptor kinase were not detected during insulin stimulation of CHO cells expressing the $IR_{F1146}$. Moreover, the $IR_{F1146}$ did not internalize insulin rapidly or stimulate DNA synthesis in the presence of insulin. In contrast, both the IR and $IR_{F1146}$ stimulated glycogen synthase equally in CHO cells. These data suggest that activation of the IR tyrosine kinase can be resolved into two components: the first is dependent on insulin binding and the second is dependent on the subsequent insulin-stimulated autophosphorylation cascade. Thus, at least two signal transduction pathways diverging from the IR are implicated in the mechanism of insulin action.

The insulin receptor (IR) kinase regulates the action of insulin on metabolism and growth through signal transduction pathways, which are only partially defined (1). The molecular link between the IR and its effector systems may involve a series of common and branching pathways, including the phosphorylation of distinct substrates (2), activation of a phosphatidylinositol kinase (3), and/or the generation of low molecular weight second messengers (4, 5). Activation of this complex regulatory network requires a functional ATP binding site in the $\beta$ subunit of the IR, suggesting that autophosphorylation of the $\beta$ subunit plays an initial role in signal transmission. At least five tyrosine phosphorylation sites have been identified in two regions of the $\beta$ subunit of the IR (6). Three of these sites, Tyr-1146, Tyr-1150, and Tyr-1151, are in the putative regulatory region; two additional sites, Tyr-1316 and Tyr-1322, are in the C terminus of the $\beta$ subunit. Autophosphorylation begins in the regulatory region immediately after insulin binding. Phosphorylation of all three tyrosine residues in the regulatory region appears to be required to fully activate the tyrosine kinase (6), whereas phosphorylation of the C terminus is not required (7).

We have prepared a mutant IR by oligonucleotide-directed mutagenesis in which Tyr-1146 has been replaced with phenylalanine ($IR_{F1146}$). Autophosphorylation of the $IR_{F1146}$ was

reduced 60–70%, which was insufficient for full activation of the receptor kinase during in vitro assays. In addition, the $IR_{F1146}$ did not internalize insulin normally or stimulate DNA synthesis in the presence of insulin. In contrast, the $IR_{F1146}$ mediated normal insulin-stimulated glycogen synthesis. These data indicate that autophosphorylation of Tyr-1146 is a critical step in the autophosphorylation cascade that activates the insulin receptor kinase; Tyr-1146 plays an essential role in insulin receptor internalization and growth regulation, but it may not be required for some metabolic signals.

## MATERIALS AND METHODS

**Oligonucleotide-Directed Mutagenesis of Human IR cDNA and Construction of Expression Plasmids.** A point mutation was introduced into the cDNA of the IR by oligonucleotide-directed mutagenesis, which replaced Tyr-1146 with phenylalanine. The template for mutagenesis was prepared by cloning a 1.9-kilobase Pst I–Pst I fragment from pCVSVHIRc (A. Ullrich, Max Planck Institute, Munich) into M13mp18 (Amersham). A uracil-rich template was prepared in Escherichia coli BW313, and mutagenesis was carried out by using the primer 5'-AGAGACATCTTCGAAACGGAT-3' (Joslin Diabetes and Endocrine Research Center Core Laboratory) by the method of Kunkel et al. (8). The mutation was confirmed by M13 dideoxy sequencing. The mutated fragment was reintroduced into pCVSVHIRc to make pCVS-VHIRc/F1146.

**Transfection of Chinese Hamster Ovary (CHO) Cells.** Subconfluent CHO cells ($10^6$) grown in F-12 medium containing 10% (vol/vol) fetal bovine serum (GIBCO) were transfected by calcium phosphate precipitation with 1 $\mu$g of pSVEneo alone or together with 10 $\mu$g of pCVSVHIRc or pCVS-VHIRc/F1146 as described (9). After 72 hr, geneticin (GIBCO) was added to the medium to select for neomycin-resistant cells. CHO cells that expressed high levels of the surface IR were selected by fluorescence-activated cell sorting (10), and clonal cell lines were obtained by plating at limiting dilution.

**[$^{35}$S]Methionine and [$^{32}$P]Phosphate Labeling of CHO Cells Expressing the Wild-Type IR (CHO/IR Cells) and the $IR_{F1146}$ (CHO/$IR_{F1146}$ Cells).** Confluent monolayers of transfected CHO cells were labeled for 24 hr with 0.5 mCi (1 Ci = 37 GBq) of [$^{35}$S]methionine or for 2 hr with 0.5 mCi of [$^{32}$P]phosphate (New England Nuclear) as described in Fig. 1 (11).

**Autophosphorylation and Kinase Activity of the Purified IR.** IR, partially purified on wheat germ agglutinin (WGA)-agarose (6), were incubated at 23°C for 30 min in 50 mM Hepes (pH 7.8) containing 5 mM $MgCl_2$ and 5 mM $MnCl_2$ in the absence or presence of 100 nM insulin. Autophosphory-

Abbreviations: IR, insulin receptor(s); $IR_{F1146}$, insulin receptor with phenylalanine substituted for tyrosine-1146; $\alpha$PY, anti-phosphotyrosine antibody; BSA, bovine serum albumin; WGA, wheat germ agglutinin.

lation was initiated by the addition of 100 $\mu$M [$\gamma$-$^{32}$P]ATP (3 $\mu$Ci/nmol; New England Nuclear). After 5 min the reaction was terminated by the addition of 2× Laemmli sample buffer containing 100 mM dithiothreitol, and the proteins were separated by SDS/PAGE (11).

The kinase activity of the IR was determined with a synthetic substrate called Thr-12-Lys, which contains amino acid residues 1143–1152 (Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys; D. Coy, Tulane University, New Orleans) of the human IR, as described (6, 7). The WGA-purified IR or IR$_{F1146}$ (4 $\mu$g) was diluted to 50 $\mu$l with 50 mM Hepes (pH 7.4) containing 5 mM MnCl$_2$ and 0.1% Triton X-100. Basal kinase activity, insulin-stimulated kinase activity, and auto-phosphorylation-stimulated kinase activity were determined as described in Fig. 3.

**Internalization of Surface-Bound Insulin.** Confluent mono-layers of CHO/IR and CHO/IR$_{F1146}$ cells were washed in phosphate-buffered saline and incubated at 4°C for 3 hr in F-12 medium containing 50 mM Hepes (pH 7.4), 0.1% bovine serum albumin (BSA), and 50,000 cpm of $^{125}$I-labeled insulin ($^{125}$I-insulin) (Amersham) per ml. After washes at 4°C, inter-nalization was initiated by the addition at 37°C of F-12 medium containing 0.1% BSA. At time intervals, the cells were acid washed (pH 3.5) at 0°C; the radioactivity retained in the cells represents internalized insulin. The cells were solubilized in 0.1% SDS/0.1 M NaOH and the $^{125}$I was measured in a $\gamma$ counter. Internalization is expressed as percent of insulin initially bound to the cell surface.

**Glycogen Synthase Assay.** Confluent CHO cells were incu-bated for 3 hr at 37°C in serum-free F-12 medium without glucose (GIBCO) prior to insulin addition at the indicated concentrations for 60 min at 37°C. The medium was removed; cells were scraped from the plates in 100 mM NaF containing 10 mM EDTA and 1 $\mu$M leupeptin, collected by centrifuga-tion, and disrupted by sonication. Portions of the cytosol extract were incubated at 30°C for 30 min with 0.5 M Tris (pH 7.8) containing 0.25 M NaF, 0.1 M EDTA, 100 mg of glycogen per ml, 36 mg of UDP-glucose per ml, and 1.6 $\mu$Ci of [$^{14}$C]UDP-glucose (DuPont/NEN) per ml. Glycogen was precipitated from the reaction mixture on 3MM Whatman

paper, the paper was immersed and washed in 66% ethanol, and $^{14}$C was measured in a scintillation counter (12). Glyco-gen synthase extracted from CHO cells behaved as previ-ously reported (13). Insulin stimulation is reported as the relative activity of glycogen synthase above the basal mea-sured in the absence of insulin and glucose-6-phosphate.

**Thymidine Incorporation.** CHO cells were incubated for 24 hr in F-12 medium containing 1% BSA without fetal bovine serum. The cells were washed and incubated for an additional 16 hr with the indicated concentrations of insulin in F-12 medium containing 1% BSA and then incubated for 1 hr at 37°C in F-12 medium containing 1% BSA, 20 mM Hepes (pH 7.4), and 1.0 mCi of [$^3$H]thymidine (New England Nuclear) per ml. The incorporation of [$^3$H]thymidine was determined as described (9).

## RESULTS

**Expression of the Normal IR and Mutant IR$_{F1146}$ in CHO Cells.** CHO/neo cells, CHO cells expressing only pSVEneo, contained ≈3000 hamster insulin receptors. After transfec-tion and selection by fluorescence-activated cell sorting, clonal lines of CHO/IR cells and mutant CHO/IR$_{F1146}$ cells were obtained, which expressed 80,000 and 75,000 receptors per cell, respectively (data not shown). Analysis of insulin binding data with a two-site model indicated high-affinity binding constants of 0.6 ± 0.3 nM (mean ± SD) and 0.4 ± 0.2 nM and low-affinity binding constants of 85 ± 40 nM and 40 ± 30 nM for the IR and IR$_{F1146}$, respectively. Thus, replace-ment of Tyr-1146 with phenylalanine did not affect the affinity of the insulin receptor.

To determine the structure of these receptors, control CHO cells (CHO/neo) or CHO cells expressing the wild-type IR and mutant IR$_{F1146}$ were labeled with [$^{35}$S]methionine for 24 hr, and the Tyr($P$)-containing proteins were immunopre-cipitated with the anti-phosphotyrosine antibody ($\alpha$PY) and analyzed by SDS/PAGE. In the absence of insulin, minor background proteins were detected in these cell lines (Fig. 1A, lanes a, c, and e). After insulin stimulation, the $\alpha$ and $\beta$ subunits were detected at 135 and 95 kDa, respectively, in
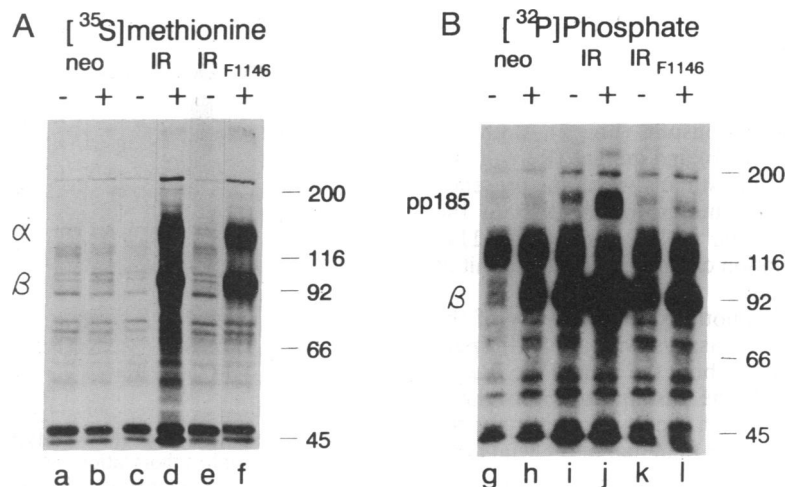


FIG. 1. Immunoprecipitation of [$^{35}$S]methionine-labeled and $^{32}$P-labeled insulin receptor with $\alpha$PY antibody. (*A*) CHO/neo cells (lanes a and b), CHO/IR cells (lanes c and d), and CHO/IR$_{F1146}$ cells (lanes e and f) were labeled with [$^{35}$S]methionine for 24 hr and then incubated in the absence (lanes a, c, and e) or presence (lanes b, d, and f) of 100 nM insulin for 1 min at 37°C. The cells were solubilized, immunoprecipitated with $\alpha$PY, and analyzed by reducing SDS/PAGE and autoradiography. The labeling was quantified by excising the $\alpha$- and $\beta$-subunit bands from the gel and determining the [$^{35}$S]methionine incorporation. No radioactivity above background was detected with the CHO/neo cells. Lane d, $\alpha$ = 2543 cpm and $\beta$ = 5178 cpm; lane f, $\alpha$ = 1431 cpm and $\beta$ = 3250 cpm. (*B*) CHO/neo cells (lanes g and h), CHO/IR cells (lanes i and j), and CHO/IR$_{F1146}$ cells (lanes k and l) were labeled with [$^{32}$P]phosphate for 2 hr and then incubated in the absence (lanes g, i, and k) or presence (lanes h, j, and l) of 100 nM insulin for 1 min at 37°C. The cells were then solubilized, immunoprecipitated with $\alpha$PY, and analyzed by reducing SDS/PAGE and autoradiography. The radiogram was purposely over exposed to allow detection of pp185. The radioactivity found in the $\beta$ subunit at the 95-kDa region of each lane was measured by Cerenkov counting: lane g, 23 cpm; lane h, 174 cpm; lane i, 761 cpm; lane j, 5556 cpm; lane k, 115 cpm; lane l, 978 cpm. Molecular size markers (in kDa) are shown at right.

CHO/IR and CHO/IR$_{F1146}$ cells (Fig. 1A, lanes b and d). The radioactivity in the $\alpha$ subunit was 50% of that in the $\beta$ subunit, which is consistent with the 1:2 ratio of methionine residues in $\alpha$ and $\beta$ subunits, respectively (14).

**Autophosphorylation of the Wild-Type IR and Mutant IR$_{F1146}$ and the Phosphorylation of pp185 in CHO Cells.** In the absence of insulin, tyrosine autophosphorylation of the rodent insulin receptor in [$^{32}$P]phosphate-labeled CHO/neo cells was nearly undetectable by immunoprecipitation with $\alpha$PY; however, after a 1-min stimulation with insulin, autophosphorylation of the $\beta$ subunit increased 7.5-fold (Fig. 1B, lanes g and h). Basal autophosphorylation of the $\beta$ subunit was increased in CHO/IR and CHO/IR$_{F1146}$ cells, and insulin stimulated autophosphorylation 7.3- and 8.5-fold, respectively. During insulin stimulation, $\beta$-subunit autophosphorylation was elevated more than 20-fold in CHO/IR cells when compared to CHO/neo cells, which is consistent with the increased number of normal human receptors in CHO/IR cells. In contrast, insulin-stimulated phosphorylation of the IR$_{F1146}$ was decreased 80%, even though the CHO/IR$_{F1146}$ cells expressed the same number of receptor molecules.

To estimate the stoichiometry of tyrosine autophosphorylation of the IR and IR$_{F1146}$, immunoprecipitation of the $\beta$ subunit by $\alpha$PY from $^{32}$P-labeled cells and [$^{35}$S]methionine-labeled cells was compared. Immunoprecipitation of the [$^{35}$S]methionine-labeled IR$_{F1146}$ from insulin-stimulated cells decreased 46% relative to the wild-type IR (Fig. 1A, lanes d and f), compared with the 80% decrease in $^{32}$P$_i$ incorporation (Fig. 1B, lanes j and l). Therefore, the decrease in the $^{32}$P$_i$ incorporation into the IR$_{F1146}$ was due partly to a decrease in the level of autophosphorylation of each mutant receptor molecule, rather than solely due to a decrease in the number of receptors undergoing autophosphorylation.

An endogenous substrate of the insulin receptor, pp185, was detected in [$^{32}$P]phosphate-labeled CHO/IR cells after 1 min of insulin stimulation (Fig. 1B). The identity and function of pp185 are unknown, but it is found in nearly all cell types immediately after insulin stimulation (2). The phosphorylation of pp185 was barely detected in CHO/neo and CHO/IR$_{F1146}$ cells, suggesting that Tyr-1146 is necessary for maximal phosphorylation of this cellular substrate.

**Kinase Activity of the Partially Purified IR and Mutant IR$_{F1146}$.** To assess autophosphorylation of IR$_{F1146}$ *in vitro*, equal concentrations of WGA-purified IR from CHO/IR and CHO/IR$_{F1146}$ cells were autophosphorylated for 5 min in the absence or presence of 100 nM insulin (Fig. 2). Both the wild-type IR (Fig. 2, lanes a and b) and mutant IR$_{F1146}$ (Fig. 2, lanes c and d) showed a 3-fold stimulation in the presence of 100 nM insulin; however, autophosphorylation of the IR$_{F1146}$ was reduced 70%. Thus, replacement of Tyr-1146 reduced the autophosphorylation cascade in the $\beta$ subunit of the IR both *in vivo* and *in vitro*.

Insulin stimulates the phosphotransferase activity of the insulin receptor during *in vitro* assays with synthetic peptides. Before insulin stimulation, both the IR and the IR$_{F1146}$ catalyzed phosphorylation of the insulin receptor-related peptide Thr-12-Lys (15). Although the basal activity of the mutant receptor was slightly lower than that of the wild type, the phosphorylation of Thr-12-Lys was stimulated 3- to 4-fold when Thr-12-Lys and [$\gamma$-$^{32}$P]ATP were added simultaneously to the insulin-stimulated IR or IR$_{F1146}$ (Fig. 3A). Under these conditions, insulin stimulation, but not the ability of autophosphorylation to further activate the receptor kinase, was measured. With the wild-type IR, the phosphotransferase activity was enhanced 12-fold by insulin-stimulated autophosphorylation carried out by addition of [$\gamma$-$^{32}$P]ATP for 20 min before addition of 1 mM Thr-12-Lys (Fig. 3A). In contrast, insulin-stimulated autophosphorylation of the mutant IR$_{F1146}$ carried out for 1, 5, 10, or 20 min before addition of Thr-12-Lys had no effect on the phosphorylation of the
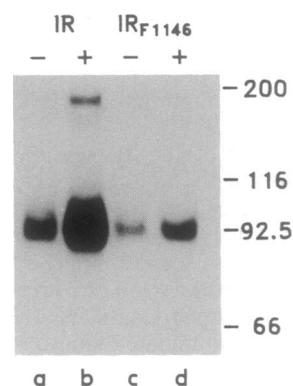


FIG. 2. *In vitro* autophosphorylation of IR and IR$_{F1146}$. WGA-purified IR (lanes a and b) and IR$_{F1146}$ (lanes c and d) were adjusted to equal insulin binding and were incubated for 30 min at 23°C in either the absence (lanes a and c) or presence (lanes b and d) of 100 nM insulin. The receptors were autophosphorylated and analyzed by reducing SDS/PAGE and autoradiography. [$^{32}$P]Phosphate incorporated into the $\beta$ subunit was quantified by Cerenkov counting of the excised $\beta$-subunit band: lane a, 504 cpm; lane b, 1146 cpm; lane c, 70 cpm; lane d, 151 cpm. Molecular size markers (in kDa) are shown at right.

peptide (Fig. 3B). Thus, mutation of the insulin receptor by the replacement of Tyr-1146 with phenylalanine separated activation of the tyrosine kinase into two components: the first is dependent on insulin binding and the second is dependent on insulin-stimulated autophosphorylation.

**Internalization of IR$_{F1146}$.** The insulin receptor is a mobile tyrosine kinase that undergoes accelerated endocytosis during insulin stimulation (16). The internalization of a single cohort of bound insulin provides a measure of the initial translocation rate of the occupied receptor (17). To quantitate internalization, CHO/IR and CHO/IR$_{F1146}$ cells were incubated with 0.1 nM $^{125}$I-insulin at 4°C for 3 hr, after which the unbound insulin was washed away and the cells were incubated at 37°C. The CHO/IR cells internalized 35% of the $^{125}$I-insulin by 15 min, whereas the CHO/IR$_{F1146}$ internalized
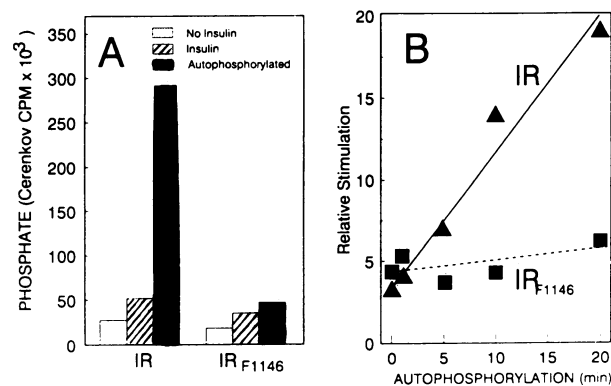


FIG. 3. Activation of the wild-type and mutant insulin receptor kinase by autophosphorylation. (A) Basal kinase activity was measured by the simultaneous addition of Thr-12-Lys peptide and [$\gamma$-$^{32}$P]ATP at final concentrations of 1 mM and 50 $\mu$M, respectively, to the IR and IR$_{F1146}$ for 5 min (No Insulin). Insulin-stimulated activity was measured in the same way following a 30-min incubation of the IR and IR$_{F1146}$ with 100 nM insulin (Insulin). The effect of autophosphorylation on kinase activity was measured by incubating the insulin-stimulated IR and IR$_{F1146}$ with [$\gamma$-$^{32}$P]ATP for 20 min followed by 1 mM Thr-12-Lys for 5 min (Autophosphorylated). (B) The IR and IR$_{F1146}$ were stimulated with 100 nM insulin for 30 min, incubated with 50 $\mu$M [$\gamma$-$^{32}$P]ATP for the designated time intervals, followed by addition of 1 mM Thr-12-Lys for 5 min. IR and IR$_{F1146}$ kinase activity toward Thr-12-Lys is expressed as relative stimulation above the basal activity (No Insulin) shown in A.
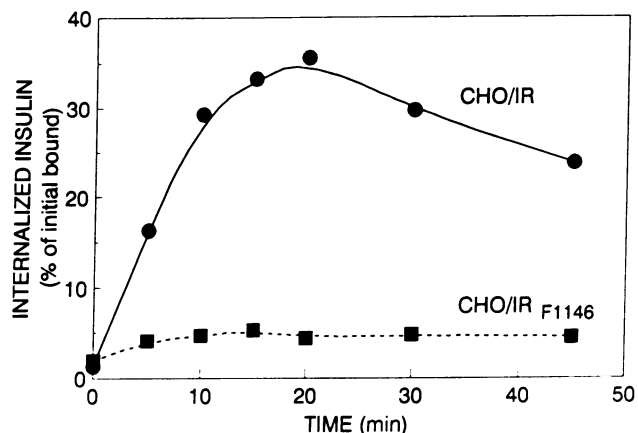
Biochemistry: Wilden et al.

Proc. Natl. Acad. Sci. USA 87 (1990)     3361



FIG. 4.   Internalization of insulin into CHO cells expressing IR and $IR_{F1146}$. CHO/IR and CHO/$IR_{F1146}$ cells were allowed to bind 0.1 nM $^{125}$I-insulin for 3 hr at 4°C. Unbound insulin was washed off, and the cells were incubated for various times at 37°C. At each time point, surface-bound external ligand was removed by washing the cells at pH 3.5, and the cells were solubilized. The acid-resistant radioactivity is defined as the amount of internalized insulin. Internalization is expressed as a percentage of initial binding.

about 5% of the $^{125}$I-insulin (Fig. 4). This loss of internalization was similar to that observed with the kinase-deficient IR in which the lysine residue in the ATP binding site was substituted with other amino acids (data not shown). Thus, replacement of Tyr-1146 with phenylalanine prevented the normal interaction between the insulin-stimulated receptor and the endocytotic machinery.

**Biological Activity of the $IR_{F1146}$.** The insulin sensitivity of CHO/IR and CHO/$IR_{F1146}$ cells was compared to the sensitivity of CHO/neo cells. Insulin stimulation of glycogen synthase was used to measure the metabolic response, and thymidine incorporation into DNA was used to measure growth regulation. Insulin stimulated glycogen synthase with
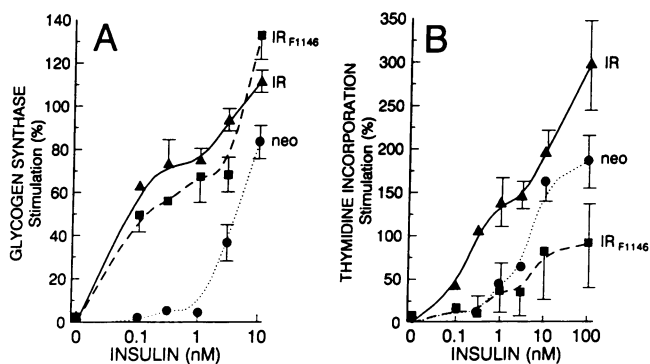


FIG. 5.   Insulin stimulation of glycogen synthase and DNA synthesis in CHO cells expressing IR and $IR_{F1146}$. The glycogen synthase activity (A) in transfected CHO cells was measured after 1 hr of insulin stimulation, and the incorporation of thymidine into DNA (B) was measured after 16 hr of insulin stimulation. The results are expressed as the percent stimulation above basal. Glycogen synthase activity and thymidine incorporation were measured in three independent experiments with similar results each time; the error bars represent the standard deviation when it is larger than the data points. For glycogen synthase, the basal activity was 375 cpm (0.35 nmol of UDP-glucose·mg$^{-1}$·min$^{-1}$), 481 cpm (0.44 nmol of UDP-glucose·mg$^{-1}$·min$^{-1}$), and 458 cpm (0.42 nmol of UDP-glucose·mg$^{-1}$·min$^{-1}$) for CHO/IR, CHO/$IR_{F1146}$ and CHO/neo cells, respectively. Each point represents an average of six determinations. For thymidine incorporation, the basal incorporation was 1762 cpm, 2712 cpm, and 2561 cpm for CHO/IR, CHO/$IR_{F1146}$, and CHO/neo cells, respectively. Each point represents an average of three determinations.

an $ED_{50}$ of ≈3 nM in CHO/neo cells (Fig. 5A). CHO/IR and CHO/$IR_{F1146}$ cells, expressing about 25 times more receptors, were ≈100-fold more sensitive to insulin. Thus the ability of the $IR_{F1146}$ to stimulate glycogen synthase was identical to the wild-type IR and was correlated with the increase in receptor number.

The effect of insulin on the incorporation of [$^3$H]thymidine into DNA of CHO/neo, CHO/IR, and CHO/$IR_{F1146}$ cells was assessed as a measure of growth response. Insulin stimulated the incorporation of thymidine into CHO/neo cells 3-fold over basal, with an $ED_{50}$ between 3 and 10 mM (Fig. 5B). The maximum responsiveness of CHO/IR cells to insulin was 4-fold the basal incorporation, and the $ED_{50}$ was between 1 and 3 nM (Fig. 5B). However, the CHO/$IR_{F1146}$ cells were less responsive to insulin than the CHO/neo cells, and the $ED_{50}$ was between 3 and 10 nM. These data suggest that replacement of Tyr-1146 impaired the activation of the mitogenic signal transduction pathway regulated by insulin.

## DISCUSSION

The tyrosine kinase activity of the IR is critical for insulin action. The kinase-deficient IR is biologically inactive (18, 19), and mutations that alter kinase activity cause some forms of human diabetes (20, 21). However, the molecular connections between the IR and cellular enzymes regulating metabolism and growth are undefined.

Immediately after insulin binding, the $\beta$ subunit of the IR undergoes autophosphorylation on at least five tyrosine residues in the $\beta$ subunit: Tyr-1146, Tyr-1150, and Tyr-1151 in the so-called regulatory region and Tyr-1316 and Tyr-1322 in the C terminus (6). Autophosphorylation of the regulatory region causes conformational changes in the $\beta$ subunit (22) and activates the tyrosine kinase (6, 22–24). Transmission of the insulin signal may occur by tyrosine phosphorylation of cellular proteins by the activated insulin receptor (9). Therefore, we predicted that replacement of Tyr-1146 with phenylalanine would diminish autophosphorylation and phosphotransferase activity of the IR and alter biological activity. Indeed, with this mutant, insulin-stimulated DNA synthesis and receptor endocytosis are reduced; however, we were surprised to find that insulin stimulated glycogen synthase normally through this mutant receptor. Thus, at least two signal transduction pathways branching from the IR, one leading to glycogen synthase activation and one leading to the DNA synthesis, are implicated in the mechanism of insulin action.

Our previous work demonstrated that insulin-stimulated autophosphorylation occurs by an ordered reaction, which begins in the regulatory region of the $\beta$ subunit (6). $\alpha$PY inhibits this cascade by binding to Tyr(P)-1146 and either Tyr(P)-1150 or Tyr(P)-1151 immediately after bisphosphorylation (6). Autophosphorylation of Tyr-1150 and Tyr-1151 appears to be random in the presence of $\alpha$PY, suggesting that Tyr-1146 is the first autophosphorylation site in the IR $\beta$ subunit (6). Replacement of Tyr-1146 with phenylalanine reduced insulin-stimulated autophosphorylation 70–80%, supporting the theory that Tyr-1146 plays an important role in the autophosphorylation cascade. Although autophosphorylation is reduced 80% in the CHO/$IR_{F1146}$ cells, more than 50% of the [$^{35}$S]methionine-labeled $IR_{F1146}$ molecules are immunoprecipitated with the $\alpha$PY from insulin-stimulated cells. This discordant result may be due to a decrease in serine and threonine phosphorylation of the $IR_{F1146}$; however, the important point is that most of the mutant receptors are still phosphorylated on at least one tyrosine residue that can be recognized by $\alpha$PY. The location of the remaining phosphorylation site(s) is unknown. The fact that insulin stimulates autophosphorylation indicates that normal communication exists between the $\alpha$ and mutant $\beta$ subunits.

Insulin binding partially activates the phosphotransferase in the mutant $IR_{F1146}$; however, the absence of the full autophosphorylation cascade apparently prevents further activation that ordinarily occurs during autophosphorylation (6, 23). Partial activation is apparently insufficient to stimulate the phosphorylation of pp185, an endogenous substrate of the IR. Insulin binding to the $IR_{F1146}$ stimulates glycogen synthase normally in CHO cells, whereas it has no effect on thymidine incorporation. Thus, phosphorylation of pp185 is not required to activate glycogen synthase, but it may be needed for DNA synthesis.

Internalization of the $IR_{F1146}$ is significantly reduced during insulin stimulation even though the $\beta$ subunit undergoes autophosphorylation on at least one tyrosine residue and the $IR_{F1146}$ shows some insulin-stimulated phosphotransferase activity. Internalization is thought to require autophosphorylation, as endocytosis of the kinase-deficient receptor is not stimulated by insulin (19, 25). However, Backer *et al.* (26) demonstrated that the IR in Fao hepatoma cells internalizes normally when autophosphorylation is reduced 90% by lowering the cellular concentration of ATP 90% with dinitrophenol. Thus, insulin-stimulated receptor internalization requires a signal that is blocked in both the kinase-deficient IR and the $IR_{F1146}$ but not by reduced autophosphorylation of the wild-type receptor.

The function of the $IR_{F1146}$ contrasts with the function of the mutant IR molecules that contain phenylalanine substitutions at the adjacent vicinal tyrosine residues 1150 and 1151. The $IR_{F1150}$ and the double mutant $IR_{F1150,F1151}$ show 3-fold elevated basal autophosphorylation during *in vitro* assays and little if any insulin stimulation (27). In contrast, basal autophosphorylation of the $IR_{F1146}$ in our experiments is inhibited 6-fold, and autophosphorylation is stimulated 4-fold. These differences may reflect the different role(s) that these residues play in the autophosphorylation cascade. The $IR_{F1150}$ does not stimulate 2-deoxyglucose uptake into CHO cells. Moreover, the mutant $IR_{F1150,F1151}$ does not support insulin stimulation of either 2-deoxyglucose uptake or glycogen synthase, whereas it stimulates normally thymidine incorporation (28). Thus the role of Tyr-1150 and Tyr-1151 in insulin signal transmission is strikingly different from the role of Tyr-1146.

In conclusion, mutation of the IR by replacement of Tyr-1146 with phenylalanine has induced a series of changes in the behavior of the insulin receptor. The $IR_{F1146}$ retains a low level of insulin-stimulated tyrosyl kinase activity but lacks the ability to undergo full activation ordinarily attributed to autophosphorylation and the ability to catalyze phosphorylation of pp185. This absence of full activation and substrate phosphorylation correlates with the loss of insulin-stimulated internalization and stimulation of thymidine incorporation but not with the stimulation of glycogen synthase. These data suggest that several signal transduction pathways branch directly from the insulin receptor to regulate these and other biological responses. Mutation in the $\beta$ subunit of the insulin receptor appears to alter selectively the interaction of the IR with these pathways.

1. Kahn, C. R. & White, M. F. (1988) *J. Clin. Invest.* **82**, 1151–1156.
2. White, M. F., Maron, R. & Kahn, C. R. (1985) *Nature (London)* **318**, 183–186.
3. Ruderman, N., Kapeller, R., White, M. F. & Cantley, L. C. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1411–1415.
4. Cheng, K. & Larner, J. (1985) *Annu. Rev. Physiol.* **47**, 405–424.
5. Saltiel, A. R. & Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5793–5797.
6. White, M. F., Shoelson, S. E., Keutmann, H. & Kahn, C. R. (1988) *J. Biol. Chem.* **263**, 2969–2980.
7. Goren, H. J., White, M. F. & Kahn, C. R. (1987) *Biochemistry* **26**, 2374–2381.
8. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382.
9. White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Dull, T. J., Ullrich, A. & Kahn, C. R. (1988) *Cell* **54**, 641–649.
10. Maron, R., Taylor, S. I., Jackson, R. & Kahn, C. R. (1984) *Diabetologia* **27**, 118–120.
11. White, M. F., Stegmann, E. W., Dull, T. J., Ullrich, A. & Kahn, C. R. (1987) *J. Biol. Chem.* **262**, 9769–9777.
12. Crettaz, M. & Kahn, C. R. (1983) *Endocrinology* **113**, 1201–1209.
13. Stalmans, W. & Hers, H. G. (1973) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), pp. 309–361.
14. 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. & Ramachandran, J. (1985) *Nature (London)* **313**, 756–761.
15. Stadtmauer, L. & Rosen, O. M. (1986) *J. Biol. Chem.* **261**, 10000–10005.
16. Backer, J. M., Kahn, C. R. & White, M. F. (1989) *J. Biol. Chem.* **264**, 1694–1701.
17. Schwartz, A. L., Fridovich, S. E. & Lodish, H. F. (1982) *J. Biol. Chem.* **257**, 4230–4237.
18. Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A. & Rosen, O. M. (1987) *J. Biol. Chem.* **262**, 1842–1847.
19. McClain, D. A., Maegawa, H., Lee, J., Dull, T. J., Ullrich, A. & Olefsky, J. M. (1987) *J. Biol. Chem.* **262**, 14663–14671.
20. Odawara, M., Kadowaki, T., Yamamoto, R., Shibasaki, Y., Tobe, K., Accili, D., Bevins, C., Mikami, Y., Matsuura, N., Akanuma, Y., Takaku, F., Taylor, S. I. & Kasuga, M. (1989) *Science* **245**, 66–68.
21. Kahn, C. R. & Goldstein, B. J. (1989) *Science* **245**, 13.
22. Herrera, R. & Rosen, O. M. (1986) *J. Biol. Chem.* **261**, 11980–11985.
23. Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M. & Cobb, M. H. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3237–3240.
24. Yu, K.-T. & Czech, M. (1984) *J. Biol. Chem.* **259**, 5277–5286.
25. Russell, D. S., Gherzi, R., Johnson, E. J., Chou, C.-K. & Rosen, O. M. (1987) *J. Biol. Chem.* **262**, 11833–11840.
26. Backer, J. M., Kahn, C. R. & White, M. F. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3209–3213.
27. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. & Rutter, W. J. (1986) *Cell* **45**, 721–732.
28. Debant, A., Clauser, E., Panzio, G., Filloux, C., Auzan, C., Contreres, J. & Rossi, B. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8032–8036.