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Mutation of the Insulin Receptor at Tyrosine 960 Inhibits Signal Transmission but Does Not Affect Its Tyrosine Kinase Activity

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Summary

Tyrosyl phosphorylation is implicated in the mechanism of insulin action. Mutation of the β-subunit of the insulin receptor by substitution of tyrosyl residue 960 with phenylalanine had no effect on insulin-stimulated autophosphorylation or phosphotransferase activity of the purified receptor. However, unlike the normal receptor, this mutant was not biologically active in Chinese hamster ovary cells. Furthermore, insulin-stimulated tyrosyl phosphorylation of at least one endogenous substrate (pp185) was increased significantly in cells expressing the normal receptor but was barely detected in cells expressing the mutant. Therefore, β-subunit autophosphorylation was not sufficient for the insulin response, and a region of the insulin receptor around Tyr-960 may facilitate phosphorylation of cellular substrates required for transmission of the insulin signal.

Introduction

Activation of the protein-tyrosine kinase in the β-subunit of the insulin receptor may be the initial molecular event in the stimulation of cellular metabolism and growth by insulin (White and Kahn, 1986; Rosen, 1987). Inactivation of the kinase by replacement of the lysine residue in the ATP-binding site with other amino acids entirely inhibits autophosphorylation and kinase activity and abolishes the insulin response (Chou et al., 1987; Ebina et al., 1987; Gherzi et al., 1987; McClain et al., 1987). Moreover, autophosphorylation of the β-subunit at Tyr-1146, Tyr-1150, and Tyr-1151 (numbered according to Ullrich et al., 1985) activates the phosphotransferase (White et al., 1988), and mutation of these residues inhibits receptor bioactivity, suggesting that autophosphorylation plays an important role in insulin action (Ellis et al., 1986). Although many details of the enzyme's activation are understood, subsequent steps in the mechanism of insulin action are poorly described. One pathway may involve the phosphorylation of cellular substrates on tyrosyl residues by the activated insulin receptor. Several putative substrates have been identified in the intact cell (White et al., 1985a; Haring et al., 1987; Perrotti et al., 1987; Bernier et al., 1987), including a protein which we call pp185 that is immunoprecipitated from insulin-stimulated cells with antiphosphotyrosine (α-PY) antibodies (White et al., 1987; Izumi et al., 1987; Kadowaki et al., 1987).

A region of the β-subunit surrounding tyrosyl residue 960 appears to be important for receptor function because antibodies that specificially bind to it inhibit insulin-stimulated autophosphorylation (Herrera et al., 1985). Tyr-960 may be an autophosphorylation site since synthetic peptides that include the amino acid sequence around Tyr-960 are phosphorylated by the purified insulin receptor (Stadtmauer and Rosen, 1986), some tryptic phosphopeptides obtained from the phosphorylated β-subunit have M_r values expected for phosphorylation at Tyr-960 (Tornqvist et al., 1987, 1988), and Tyr-960 is phosphorylated in truncated receptor molecules (Herrera et al., 1988).

To examine the role of Tyr-960 in the β-subunit directly, we have prepared a mutation using oligonucleotide-directed mutagenesis to substitute phenylalanine at this position, and expressed this mutant cDNA in Chinese hamster ovary (CHO) cells. Insulin binding and autophosphorylation of the mutant receptor were normal; however, the mutant did not stimulate tyrosyl phosphorylation of pp185 and did not stimulate glycogen synthase, amino acid uptake, or thymidine incorporation. We conclude that Tyr-960 is not a site of autophosphorylation in the intact receptor, but this residue is required for signal transmission following insulin binding. We suggest that molecular events in addition to tyrosine autophosphorylation, possibly the phosphorylation of cellular substrates such as pp185, may be necessary for the biological activity of insulin.

Results

Tyr-960 Is Not an Autophosphorylation Site and Is Not Important for Phosphotransferase Activation in the Partially Purified Insulin Receptor

CHO cells transfected with normal (CHO/$HIRC_1$) or mutant (CHO/$F960_2$) human insulin receptor cDNA expressed 10 times more insulin binding sites than did control cells (CHO/NEO). However, the replacement of Tyr-960 with phenylalanine did not affect the affinity of insulin binding (data not shown). In the absence of insulin, autophosphorylation of the purified receptors was nearly undetectable during incubation with [γ-^{32}P]ATP (Figure 1, lanes a, c, and e). After insulin stimulation, the autophosphorylation of the β-subunit in each preparation was significantly increased (Figure 1, lanes b, d, and f). Autophosphorylation of the receptor from CHO/$HIRC_1$ and CHO/$F960_1$ cells was about 7- to 8-fold higher than autophosphorylation of the receptor from CHO/NEO cells, consistent with the increased number of receptors.

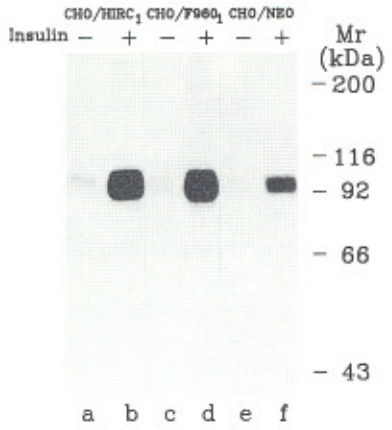


Figure 1. Phosphorylation of the β-Subunit of the Insulin Receptor Purified from Transfected CHO Cells

The insulin receptor was purified partially from the indicated cell lines, and 2 μg of purified protein was incubated in the absence (–) or presence (+) of 100 nM insulin for 20 min at 22°C. Phosphorylation was initiated by adding 25 μM [γ-^{32}P]ATP for 30 min. The reaction was terminated, and the phosphorylated receptor was immunoprecipitated with α-PY antibody, reduced with DTT, and separated by SDS-PAGE (see Experimental Procedures). An autoradiogram obtained after a 5 hr exposure is shown. Cerenkov cpm in each band: CHO/NEO = 1,573 cpm; CHO/HIRC$_1$ = 9,921 cpm; CHO/F960$_1$ = 10,633 cpm.

The tryptic phosphopeptide map of the mutant insulin receptor labeled in vitro with [γ-^{32}P]ATP during insulin stimulation was identical to that of the normal receptor (Figures 2A and 2B). The structures of the tryptic phosphopeptides were deduced previously and indicate that pY1 and pY1a each contain Tyr(P)-1146, Tyr(P)-1150, and Tyr(P)-1151, pY4 contains Tyr(P)-1146 and either Tyr(P)-1150 or Tyr(P)-1151, whereas pY2 and pY3 are derived from the C-terminus and contain Tyr(P)-1316 and Tyr(P)-1322 (White et al., 1988). Tyr-960 is not a major site of autophosphorylation since no peaks were missing from the high-pressure liquid chromatography (HPLC) profile of the mutant receptor. Moreover, substitution of Tyr-960 with phenylalanine did not alter autophosphorylation of the major sites in the partially purified insulin receptor.

Insulin-stimulated autophosphorylation of the β-subunit increases the phosphotransferase activity of the purified insulin receptor toward exogenous substrates such as the dodecapeptide Thr-12-Lys (see Experimental Procedures; Rosen et al., 1983; Yu and Czech, 1984). However, high concentrations of substrates inhibit autophosphorylation, which blocks the activation of the phosphotransferase and causes biphasic kinetic curves (Kwok et al., 1986; White et al., 1988). In the absence of insulin stimulation and prior autophosphorylation, the V_{max} for phosphorylation of Thr-12-Lys by the normal and mutant insulin receptors was about 0.05 pmol/min (Figure 3). By contrast, after insulin-stimulated autophosphorylation of the normal and mutant receptors for 5 min, the phosphotransferase was stimulated more than 10-fold during assays at concentrations of Thr-12-Lys below 1 mM. Above 1 mM Thr-12-Lys, a portion of the stimulation was inhibited because high substrate concentrations inhibit additional autophosphorylation that occurs ordinarily during the kinase assay (Figure 3). Thus,

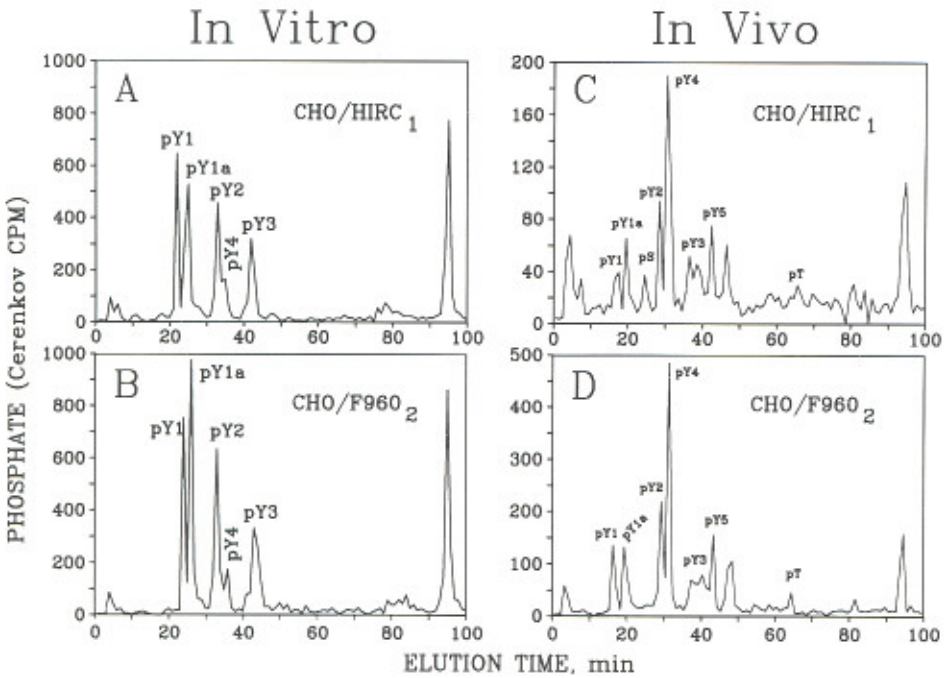


Figure 2. Separation of Tryptic Phosphopeptides from the β-Subunit of the Insulin Receptor

Purified insulin receptors in CHO/HIRC$_1$ (A) or CHO/F960$_2$ (B) cells were incubated with [γ-^{32}P]ATP and insulin; receptors from CHO/HIRC$_1$ (C) or CHO/F960$_2$ (D) cells were labeled for 2 hr with [^{32}P]phosphate before insulin stimulation. Phosphorylated receptors were immunoprecipitated with α-PY, reduced with DTT, separated by SDS-PAGE, and digested exhaustively with trypsin. The tryptic phosphopeptides were separated by reverse-phase HPLC. Phosphopeptides are labeled as described previously (White et al., 1988).

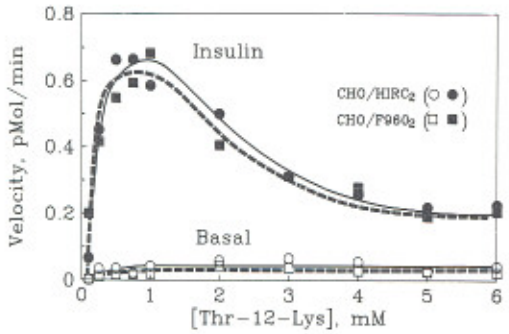


Figure 3. Phosphotransferase Activity of the Purified Insulin Receptor

The insulin receptor was purified partially from CHO/HIRC$_2$ cells (circles) and CHO/F960$_2$ cells (squares) on immobilized WGA (see Experimental Procedures). The relative amount of active receptor in each preparation was estimated by the level of insulin-stimulated autophosphorylation obtained after 60 min of incubation with 50 μM [γ-^{32}P]-ATP: CHO/HIRC = 0.9/mg protein, and CHO/F960$_2$ = 0.7/mg protein. For the kinase assay, receptor preparation (4 μg) was incubated in the absence (open symbols) or presence (closed symbols) of 100 nM insulin for 20 min at 22°C. The insulin-stimulated receptor was phosphorylated for 5 min with 50 μM [γ-^{32}P]ATP, and then the indicated concentrations of Thr-12-Lys were added to the basal and insulin-stimulated reactions. The basal reaction was initiated by adding 50 μM [γ-^{32}P]ATP. Phosphorylation of Thr-12-Lys was carried out for 5 min; the amount of [^{32}P]phosphate incorporated into Thr-12-Lys was measured by Cerenkov counting and normalized to the amount of active receptor added to the reaction.

the phosphotransferase of the mutant and normal insulin receptors was stimulated by autophosphorylation and showed identical kinetic characteristics during in vitro assays.

Effect of the Phe-960 Mutation on the Biological Activity of the Insulin Receptor

The biological activity of mutant and wild-type human insulin receptors expressed in CHO cells was evaluated by measuring the activity of glycogen synthase after insulin stimulation for 30 min, and the uptake of amino acids and the incorporation of thymidine after insulin stimulation for 2.5 hr and 15 hr, respectively. Glycogen synthase was stimulated by insulin in CHO/NEO cells, with the half-maximal effect occurring at about 5 nM (Figure 4A). CHO/HIRC$_2$ cells were about 50-fold more sensitive to insulin, since the ED$_{50}$ decreased to 0.1 nM (Figure 4A). This result was consistent with other studies showing increased sensitivity of the insulin response in cells that overexpress the normal insulin receptor (Ebina et al., 1985a; Chou et al., 1987). In contrast, the sensitivity of glycogen synthase to insulin was not increased in the CHO/F960$_2$ cells, and the maximal response reached at 100 nM insulin was decreased (Figure 4A). Similar results were found in four separate experiments using other CHO cell lines expressing the mutant receptor.

Next, we measured the ability of insulin to stimulate the uptake of α-(methylamino)isobutyric acid (MeAIB) into the transfected CHO cells. MeAIB is a specific substrate for amino acid transport system A, which is stimulated by insulin in several cell types (Shotwell et al., 1983). Insulin stimulated the specific influx of MeAIB into the control CHO/NEO cells about 60%, with a half-maximal effect at about 2 nM insulin (Figure 4B). MeAIB uptake into the CHO/HIRC$_1$ cells was increased 60% by insulin, and the cells were 10- to 20-fold more sensitive to insulin. In contrast, the sensitivity of CHO/F960$_2$ cells to insulin stimulation was equal to that of the control CHO/NEO cells, and the magnitude of the response measured at 100 nM insulin was decreased by 50% (Figure 4B). Similar results were found for the CHO/F960$_1$ cells. Thus, the mutant insulin receptor did not stimulate amino acid uptake in CHO cells.

Finally, we measured the effect of insulin on the incorporation of [^{3}H]thymidine into DNA of transfected CHO cells. Insulin stimulated the incorporation of thymidine into control CHO/NEO cells with an ED$_{50}$ of 20 nM (Figure 4C). The sensitivity to insulin was increased about 10-fold in CHO/HIRC$_2$ cells, and the magnitude of the response at 100 nM insulin increased 2-fold; however, the CHO/F960$_2$ cells expressing the mutant receptor were identical to the CHO/NEO cells. These data are consistent with the previous results indicating that replacement of Tyr-960 with phenylalanine inhibited the biological activity of the insulin receptor.

The Mutant Insulin Receptor Undergoes Autophosphorylation Normally in CHO Cells but Does Not Stimulate Tyrosyl Phosphorylation of the Endogenous Substrate pp185

The α-PY antibody recognizes the insulin receptor and putative cellular substrates during insulin stimulation (White et al., 1985a, 1987; Tornqvist et al., 1988). Our transfected CHO cells were labeled with [^{32}P]phosphate, stimulated with insulin (100 nM) for 5 min, and homogenized in the absence of Triton X-100; particulate and cytosolic fractions were prepared by centrifugation. The membrane proteins in the particulate fraction were extracted with 1% Triton X-100, and phosphotyrosine-containing proteins were immunoprecipitated with α-PY antibody (Figure 5A). Before insulin stimulation, no proteins were immunoprecipitated from the membrane fraction. However, insulin slightly stimulated the phosphorylation of a 95 kd membrane protein in CHO/NEO cells that corresponded to the β-subunit of the rodent insulin receptor (Figure 5A, lanes a and b). CHO/HIRC$_2$ cells and CHO/F960$_2$ cells displayed a 20-fold higher β-subunit phosphorylation during insulin stimulation, consistent with the increased number of receptors in these cells (Figure 5A, lanes c–f). The β-subunit of the insulin receptor was not detected during immunoprecipitation of the cytosolic extract with α-PY (Figure 5B, lanes a–f).

The HPLC profile of the β-subunit labeled in vivo differed from that obtained during in vitro labeling, but the differences were nearly identical for the normal and mutant human insulin receptors (Figures 2C and 2D). Moreover, the profiles were identical to those shown previously for Fao hepatoma cells (White et al., 1988): pY4 is the major phosphopeptide observed after in vivo labeling and contains two phosphotyrosyl residues, Tyr(P)-1146 and either Tyr(P)-1150 or Tyr(P)-1151. pY1 and pY1a, both

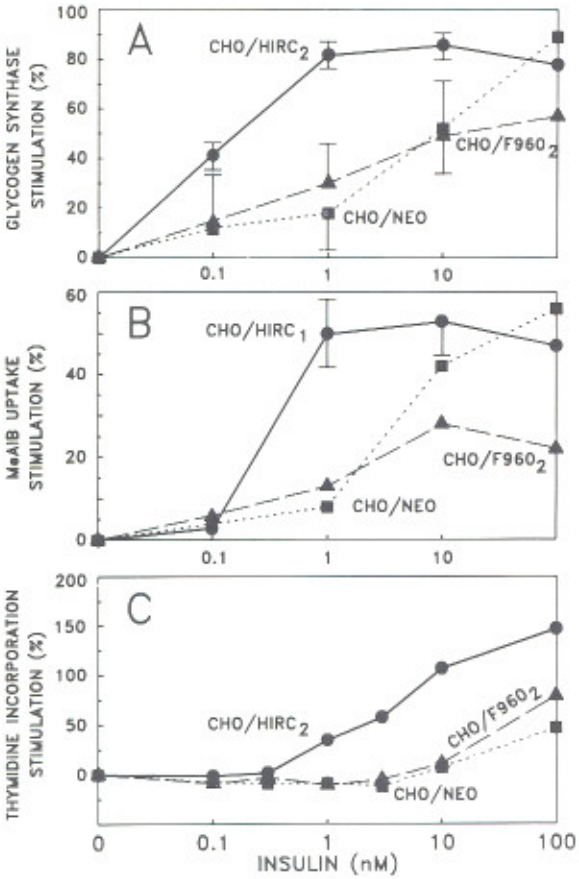


Figure 4. Biological Activity of Normal and Mutant Insulin Receptors

The activity of glycogen synthase (A) in transfected CHO cells was measured after insulin stimulation for 30 min, and the uptake of MeAIB (B) and the incorporation of thymidine (C) were measured after insulin stimulation for 2.5 hr and 15 hr, respectively. Results are expressed as the percentage stimulation above the basal activity. Each point represents an average of three or four determinations, and an error bar is shown where the standard deviation is greater than 5%.

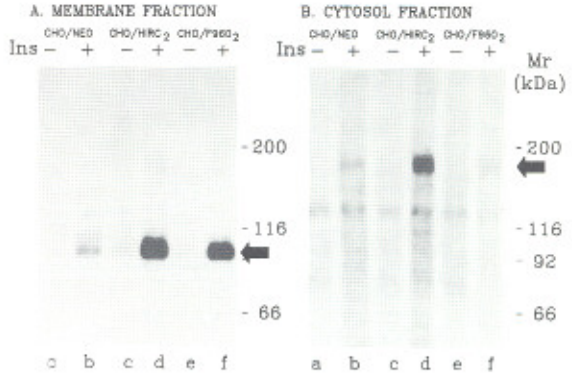


Figure 5. Immunoprecipitation of Phosphotyrosine-Containing Proteins from [^{32}P]Phosphate-Labeled CHO Cells

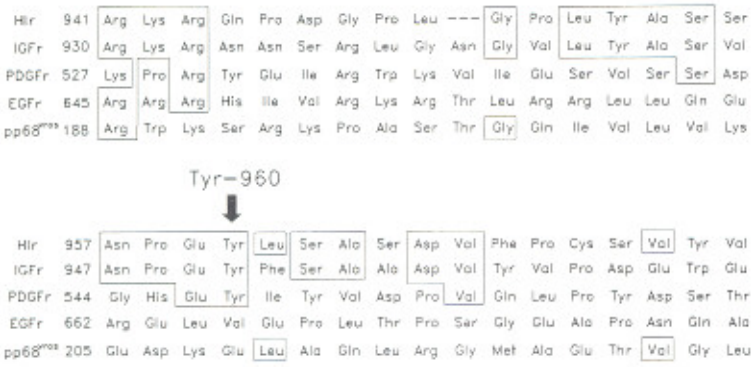
Control CHO/NEO cells (lanes a and b) or CHO/HIRC$_2$ cells (lanes c and d) and CHO/F960$_2$ cells (lanes e and f) were labeled for 2 hr with [^{32}P]orthophosphate. Cells were incubated without (−) or with (+) 100 nM insulin for 5 min, and separated into a membrane (A) and a cytosolic (B) fraction by centrifugation as described in Experimental Procedures. Phosphotyrosine-containing proteins in each fraction were immunoprecipitated with the α-PY antibody, reduced with DTT, and separated by SDS-PAGE. The autoradiogram was obtained during a 6 hr exposure, and arrows indicate the positions of the β-subunit (A) and pp185 (B).

of which contain all three phosphotyrosyl residues, were minor peaks of the in vivo peptide maps, whereas pY1 and pY1a were major peaks during in vitro labeling (Figure 2). Phosphorylation of the C-terminus (pY2 and pY3) was also found during in vivo labeling of the wild-type and mutant receptors. The insulin receptor is also phosphorylated on seryl and threonyl residues in the intact cell (White et al., 1985b; Takayama et al., 1988). One of the major phosphoserine-containing tryptic peptides (pS) was detected in the normal insulin receptor (Figure 2C), but this phosphopeptide was not detected in the mutant receptor immunoprecipitated from CHO/F960$_2$ cells (Figure 2D). Other unidentified phosphopeptides were unchanged, suggesting that mutation of the β-subunit by substitution of Tyr-960 with phenylalanine did not alter the tyrosyl phosphorylation pattern of the β-subunit in vivo.

Previous results indicated that several cell lines including CHO cells contain a putative endogenous substrate for the insulin receptor, called pp185 (White et al., 1985a). In contrast to the insulin receptor and its precursors, which are integral membrane proteins, pp185 is cytosolic or weakly membrane bound (White et al., 1987). Insulin stimulated the tyrosyl phosphorylation of a 175 kd protein that was immunopurified from cytosolic extracts of CHO/NEO cells using α-PY antibody (Figure 5B, lanes a and b). Based on previous results, this protein corresponds to pp185. Other minor bands were found in the α-PY immunoprecipitate, but they were not stimulated by insulin and their phosphorylation was not increased in CHO/HIRC cells. In contrast, the recovery of pp185 was increased 10-fold ih the CHO/HIRC$_2$ cells, whereas it was barely detected in the CHO/F960$_2$ cells (Figure 5B, lanes c–f). A small amount of pp185 was found in the solubilized membrane fraction of CHO/HIRC$_2$ cells (Figure 5A, lanes c and d). Thus, replacement of Tyr-960 in the β-subunit with phenylalanine inhibited the tyrosyl phosphorylation of pp185 during insulin stimulation.

Discussion

The β-subunit of the insulin receptor undergoes autophosphorylation on several tyrosyl residues immediately after insulin binding (White et al., 1984, 1988; Tornqvist et al., 1987), but the molecular mechanism by which the tyrosine kinase regulates cellular metabolism and growth is not understood. Autophosphorylation activates the phosphotransferase of the purified insulin receptor, suggesting that signal transduction may occur in the intact cell by a cascade of substrate phosphorylation. Therefore, we were surprised to find that substitution of Tyr-960 with phenylalanine had no effect on β-subunit autophosphorylation in vivo or in vitro and on tyrosine kinase activity in vitro, whereas the mutant receptor did not stimulate glycogen synthase, amino acid uptake, or thymidine incorporation.



Hlr	941	Arg	Lys	Arg	Gln	Pro	Asp	Gly	Pro	Leu	---	Gly	Pro	Leu	Tyr	Ala	Ser	Ser
IGFr	930	Arg	Lys	Arg	Asn	Asn	Ser	Arg	Leu	Gly	Asn	Gly	Val	Leu	Tyr	Ala	Ser	Val
PDGFr	527	Lys	Pro	Arg	Tyr	Glu	Ile	Arg	Trp	Lys	Val	Ile	Glu	Ser	Val	Ser	Ser	Asp
EGFr	645	Arg	Arg	Arg	His	Ile	Val	Arg	Lys	Arg	Thr	Leu	Arg	Arg	Leu	Leu	Gln	Glu
pp68$^{v\text{-}ros}$	188	Arg	Trp	Lys	Ser	Arg	Lys	Pro	Ala	Ser	Thr	Gly	Gln	Ile	Val	Leu	Val	Lys
Hlr	957	Asn	Pro	Glu	Tyr	Leu	Ser	Ala	Ser	Asp	Val	Phe	Pro	Cys	Ser	Val	Tyr	Val
IGFr	947	Asn	Pro	Glu	Tyr	Phe	Ser	Ala	Ala	Asp	Val	Tyr	Val	Pro	Asp	Glu	Trp	Glu
PDGFr	544	Gly	His	Glu	Tyr	Ile	Tyr	Val	Asp	Pro	Val	Gln	Leu	Pro	Tyr	Asp	Ser	Thr
EGFr	662	Arg	Glu	Leu	Val	Glu	Pro	Leu	Thr	Pro	Ser	Gly	Glu	Ala	Pro	Asn	Gln	Ala
pp68$^{v\text{-}ros}$	205	Glu	Asp	Lys	Glu	Leu	Ala	Gln	Leu	Arg	Gly	Met	Ala	Glu	Thr	Val	Gly	Leu

Figure 6. Comparison of the Primary Amino Acid Sequences of Receptor-Tyrosine Kinases

The amino acid sequences beginning just after the transmembrane spanning region are compared for the human insulin receptor (Hlr; Ullrich et al., 1985), the human type I IGF receptor (IGFr; Ullrich et al., 1986), the murine PDGF receptor (PDGFr; Yarden et al., 1986), the human EGF receptor (EGFr; Ullrich et al., 1984), and the chicken sarcoma virus UR2 transforming protein prp68$^{v\text{-}ros}$ (Matsushime et al., 1986). Tyr-960 in Hlr is indicated with an arrow, and identical residues are outlined. Numbers indicate amino acid positions. One gap was introduced in Hlr to maximize alignment.

Thus, in addition to the ATP-binding domain and the autophosphorylation sites of the β-subunit, Tyr-960 appears to be required for biological activity of the insulin receptor.

Transmission of the insulin signal may occur in cells by tyrosine phosphorylation of cellular substrates. α-PY antibodies and other approaches have identified some potential substrates for the insulin receptor in the intact cell during insulin stimulation (White et al., 1985a; Haring et al., 1987; Perrotti et al., 1987; Bernier et al., 1987; White et al., 1987; Izumi et al., 1987; Kadowaki et al., 1987). One of these, pp185, was immunoprecipitated with α-PY antibody from the cytosol fraction of insulin-stimulated CHO/NEO cells, and its recovery was 10-fold higher from CHO/HIRC cells (which contained more active receptors). However, phosphorylation of pp185 was not increased in the CHO/F960 cells when compared with the control CHO/NEO cells, suggesting that the mutant insulin receptor did not stimulate tyrosyl phosphorylation of pp185. The replacement of Tyr-960 with phenylalanine may change the conformation of the β-subunit so that pp185 is not recognized and phosphorylated by the receptor during insulin stimulation.

Although the mutant insulin receptor was expressed in CHO/F960 cells at levels equal to or greater than those of the normal receptor in CHO/HIRC cells, the mutant did not lower the ED_{50} for several biological responses. This result was unexpected since autophosphorylation of the mutant receptors was normal in vitro and in vivo. However, the absence of biological activity correlated with the inability of the mutant receptor to stimulate the tyrosyl phosphorylation of pp185. Together, these results suggest that tyrosyl phosphorylation of an endogenous substrate may mediate the biological response to insulin. We do not know the function of pp185 or its role in signal transmission. As tyrosyl phosphorylation of pp185 occurs immediately after insulin stimulation (White et al., 1987), our results suggest that pp185 directly interacts with the insulin receptor and plays an early role in transmission of the insulin signal beyond the plasma membrane. However, phosphorylation of pp185 may not be an obligatory step in the signal cascade, and its phosphorylation could be a response to the activation of another signal pathway that regulates glycogen synthase, amino acid uptake, and thymidine incorporation in parallel.

The pp185 is also phosphorylated during stimulation of the type I insulin-like growth factor (IGF) receptor (Izumi et al., 1987). This is consistent with the fact that the amino acid sequence of the β-subunit cytoplasmic region of the insulin and IGF receptors is 84% homologous (Ullrich et al., 1986). Moreover, Tyr-960 and its flanking amino sequence are conserved in the IGF receptor (Figure 6). A similar sequence is not found in the epidermal growth factor (EGF) receptor (Figure 6) and pp185 is not phosphorylated by this kinase (Kadowaki et al., 1987), suggesting that this region may form a substrate-recognition domain. Moreover, a hybrid receptor molecule composed of the extracellular ligand-binding domain of the human insulin receptor and the transmembrane and cytoplasmic domains of the chicken sarcoma virus UR2 transforming protein, pp68$^{v\text{-}ros}$, undergoes insulin-stimulated autophosphorylation. Although the catalytic domain of pp68$^{v\text{-}ros}$ is 50% identical to the β-subunit of the insulin receptor, there is no residue identical to Tyr-960 (Figure 6), and the hybrid is not biologically active in CHO cells (Ellis et al., 1987). The platelet-derived growth factor (PDGF) receptor contains a residue identical to Tyr-960, but the surrounding amino acid sequence is different (Figure 6), and a distinct set of phosphoproteins between 75 and 90 kd are immunoprecipitated from PDGF-stimulated cells using α-PY antibody (Kaplan et al., 1987). Thus, Tyr-960 and the surrounding identical amino acids of the insulin and type I IGF receptors may be an important region for recognition and phosphorylation of pp185 and other specific cellular targets, whereas differences in this region noted for the EGF and PDGF receptors and pp68$^{v\text{-}ros}$ may direct the tyrosyl phosphorylation of distinct cellular targets that mediate distinct biological actions.

Mutagenesis of protein-tyrosine kinases has focused on the ATP-binding domain and the major sites of tyrosine autophosphorylation. All protein kinases contain a homologous domain thought to be important for ATP binding that includes a lysine residue that may facilitate the nucleophilic attack of the γ-phosphate of ATP (Kamps et al., 1984). Substitution of this lysine residue with other amino acids completely inactivates the tyrosine kinases (Hunter and Cooper, 1986). In the case of the insulin receptor, autophosphorylation is blocked, phosphorylation of pp185 is eliminated, and the receptor does not

transduce a biological signal (Chou et al., 1987; Ebina et al., 1987; McClain et al., 1987). These results generally suggest that ATP binding and autophosphorylation are absolutely required for signal transmission. However, our results with the Tyr-960 mutation indicate that ATP binding and autophosphorylation are not sufficient for insulin action.

The major phosphorylation sites in the insulin receptor are located in two β-subunit regions: the C-terminus at Tyr-1316 and Tyr-1322, and the region including Tyr-1146, Tyr-1150, and Tyr-1151 (Tornqvist et al., 1987; White et al., 1988). The C-terminus is least homologous to other kinases, and its removal by mild trypsin digestion does not destroy the kinase activity of the insulin receptor (Goren et al., 1987; White et al., 1988); this region may not be necessary for biological activity (Izumi et al., 1988). In contrast, the Tyr-1150 region contains a high degree of amino acid sequence identity between the members of the protein-tyrosine kinase family (Hunter and Cooper, 1986). It contains the first sites of autophosphorylation detected during insulin stimulation of the purified receptor, and it is the predominant phosphotyrosine-containing domain of the β-subunit in insulin-stimulated cells (White et al., 1988). Phosphorylation of tyrosyl residues 1146, 1150, and 1151 activates the phosphotransferase during in vitro kinase assays (White et al., 1988). The phosphorylation of these tyrosyl residues was not affected by our mutation of the β-subunit, and insulin-stimulated autophosphorylation of these residues activated normally the phosphotransferase of the β-subunit. However, substitution of Tyr-1150 and Tyr-1151 (called Tyr-1161 and Tyr-1162, respectively, according to the numbering system of Ebina et al. [1985b]) with phenylalanine decreases by more than half the insulin-stimulated autophosphorylation of the β-subunit in the intact cell and prevents activation of the phosphotransferase in vitro (Ellis et al., 1986). This mutation also decreases sensitivity of insulin-stimulated 2-deoxyglucose uptake into transfected CHO cells. Thus, phosphorylation of the Tyr-1150 domain plays a central role in the regulation of the insulin-stimulated tyrosine kinase.

In conclusion, we have investigated the structure and function of an insulin receptor that contains a phenylalanine residue in place of Tyr-960. Autophosphorylation of the mutant β-subunit is stimulated normally by insulin during in vivo and in vitro assays, suggesting that Tyr-960 is not an autophosphorylation site. The mutant receptor mediates the internalization of insulin into CHO cells, indicating that the phenylalanine substitution does not alter the ability of the insulin receptor to accomplish some of its normal functions. However, during insulin stimulation of CHO/F960 cells, the mutant receptor does not stimulate the phosphorylation of pp185 and does not activate glycogen synthase, amino acid transport system A, or thymidine incorporation. Although tyrosine kinase activity of the receptor is required for insulin action, it appears to be insufficient, and other mechanisms involving the Tyr-960 region of the β-subunit may be required for efficient transmission of the insulin signal. One possibility is that the Tyr-960 region recognizes cellular substrates such as pp185 and facilitates their interaction with the catalytic domain of the insulin receptor, resulting in tyrosyl phosphorylation and activation of the target proteins.

Experimental Procedures

Oligonucleotide-Directed Mutagenesis of Human Insulin Receptor cDNA and Construction of Expression Plasmids

The normal human insulin receptor expression plasmid pCVSHVIRc has been previously described (McClain et al., 1987). The plasmid for the expression of the mutant insulin receptor was generated by oligonucleotide-directed mutagenesis. A BglII–HindIII fragment from pCVSVHIRc was cloned into M13mp19, and mutagenesis was carried out on a single-stranded template using the primer 5′-CTTCAAACC-CTGAGTTTCTCAGTGCCAGT (Gillam et al., 1979). Escherichia coli strain JM101 was transformed with double-stranded circular DNA, and plaques were screened under stringent conditions using the mutagenesis primer as a probe. The mutation was confirmed by M13 dideoxy sequencing (Sanger et al., 1977) and reintroduced into the pCVSVHIR vector. This new plasmid, pCVSVHIRF/Y960, replaced phenylalanine for tyrosine at position 960 of the insulin receptor precursor (Ullrich et al., 1985).

Transfection of CHO Cells

CHO cells were grown in 10 or 15 cm dishes (Nunc) containing 20 or 30 ml of F12 medium supplemented with 10% fetal bovine serum (GIBCO). The CHO cells (10^6 per 10 cm dish) were transfected by calcium phosphate precipitation with a neomycin resistance gene (pSVEneo; 2 μg) alone or together with either pCVSVHIRc or pCVSVHIRF/Y960 (10 μg) as previously described (White et al., 1987). After a 24 hr incubation, 800 μg/ml of geneticin (GIBCO) was added to the medium to select neomycin-resistant cells. Ten to 14 days later, surviving colonies were harvested and cultured in the presence of G418 to amplify the cell line.

CHO cells that expressed high levels of surface insulin receptors were selected by fluorescence-activated cell sorting (Maron et al., 1984). Sorting was repeated three to five times for each cell line used in this study before single clones were selected by limiting dilution. The level of insulin receptor expression was determined by insulin binding (White et al., 1987). Two lines of CHO cells were selected that expressed different amounts of the normal insulin receptor: CHO/HIRC$_1$—three sorts, clone k, 40,000 receptors per cell; and CHO/HIRC$_2$—five sorts, clone l, 80,000 receptors per cell. Two lines of CHO cells were selected that expressed different amounts of the mutant receptor: CHO/F960$_1$—five sorts, clone h, 40,000 receptors per cell; and CHO/F960$_2$—five sorts, clone d, 70,000 receptors per cell. A control cell line, CHO/NEO, expressed only pSVEneo and contained about 3,000 hamster insulin receptors per cell. Analysis of insulin-binding data using a two-sites model (White et al., 1987) indicated that the high-affinity binding constants (±SD) for the wild-type and mutant receptors were 0.6 ± 0.2 nM and 0.8 ± 0.1 nM, respectively; the values for the low-affinity binding constant were 79 ± 48 nM and 50 ± 25 nM, respectively.

Ribonuclease cleavage of an antisense RNA probe was used to demonstrate the expression of the mutant insulin receptor mRNA in CHO/F960 cells. A 1599 bp insert (2746 bp to 4345 bp) between two PstI sites of the normal insulin receptor cDNA was symmetrically subcloned into a pGEM-42 plasmid (Promega Biotec, Madison, WI) (Davis et al., 1986), and a full-length antisense RNA probe was prepared with [α-^{32}P]GTP (Melton et al., 1984). Using mRNA isolated from the CHO/F960$_2$ cells, this procedure detected two protected fragments of about 260 and 1350 bases corresponding to a cleavage site introduced by the altered sequence at the Tyr-960 codon site. In contrast, this cleavage was not detected with mRNA from the CHO/HIRC$_1$ cells, suggesting that all of the insulin receptor mRNA expressed in these cells was normal (B. Goldstein and C. R. K., unpublished).

Glycogen Synthase Assay

CHO cells were grown to confluence in 100 mm dishes. Prior to insulin stimulation, the cells were incubated in 4 ml of F12 medium (pH 7.4) for 3 hr at 37°C, and insulin was added at the indicated concentrations for 60 min at 37°. Cells were washed at 4°C with 3 ml of 100 mM NaF, 10 mM EDTA, and then scraped from the plates, collected

by centrifugation, and disrupted by sonication. The particulate fraction was removed by centrifugation, and glycogen synthase in the supernatant was assayed as described previously (Crettaz and Kahn, 1983). The relative stimulation (± SD) of four measurements obtained in the absence of D-glucose was reported.

Amino Acid Uptake

Confluent CHO cells in 24-well trays (Costar) were incubated in 1 ml of serum-free F12 for 24 hr and then incubated with F12 containing 0.1% bovine serum albumin (BSA) and the indicated concentrations of insulin for 2.5 hr. Cells were washed once with Krebs-Ringer bicarbonate and incubated for 30 min with this solution containing 0.1% BSA and the same concentrations of insulin to deplete the intracellular amino acids and release the system A from transinhibition (White and Christensen, 1983). Finally, the influx of tracer [^{14}C]MeAIB (New England Nuclear) and 0.1 mM MeAIB (Sigma) was measured during a 1 min time interval as previously described (Gazzola et al., 1981). The nonspecific uptake was measured in the presence of 20 mM MeAIB and subtracted from the data. The relative stimulation (± SD) of three measurements was reported.

Thymidine Incorporation

Confluent CHO cells, grown in 24-well trays (Costar), were incubated for 24 hr in 1 ml of F12 medium containing 1% BSA without fetal bovine serum. These cells were washed and incubated for an additional 18 hr with the indicated concentrations of insulin in 1 ml of F12 medium containing 1% BSA. Finally, the cells were incubated for 1 hr at 37°C in F12 medium containing 1% BSA, 20 mM HEPES (pH 7.4), and 0.5 μCi/ml [^{3}H]thymidine (New England Nuclear). The solution was removed, and cells were washed three times with ice-cold phosphate-buffered saline. The monolayers were dissolved in 1 ml of SDS (1 mg/ml), and DNA was precipitated with 2 ml of 20% trichloroacetic acid added to the extract at 4°C. The precipitate was collected by centrifugation, the pellet was suspended in 1 N NaOH and neutralized, and the radioactivity was measured in a scintillation counter using ACS scintillation cocktail (Amersham). The relative stimulation (± SD) of three measurements was reported.

In Vitro Autophosphorylation Assay

Insulin receptor (0.24 μg/μl) purified on wheat germ agglutinin (WGA)–agarose (Vector Sciences) was incubated at 22°C for 10 min in a solution (50 μl) containing 50 mM HEPES (Sigma) and 5 mM $MnCl_2$ (Sigma) in the absence or presence of 100 nM insulin (White et al., 1988). Phosphorylation was initiated by adding 25 μM [γ-^{32}P]ATP (2.5 mCi/ml, New England Nuclear) and was continued for the time intervals indicated in the figure legends. The phosphorylation reaction was terminated at 4°C by adding a 0.5 ml portion of 0.1% Triton X-100 (New England Nuclear) containing 50 mM HEPES, 100 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA (all from Sigma), and 2 mM sodium vanadate (from Aldrich). The phosphorylated insulin receptor was immunoprecipitated from each reaction with 1 μg/ml α-PY antibody prepared as previously described (Pang et al., 1985). The phosphoproteins were eluted from the Pansorbin with Laemmli sample buffer containing 100 mM dithiothreitol (DTT) separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% resolving gels, and identified by autoradiography (Crettaz et al., 1984).

Tyrosine Kinase Assay

The kinase activity of the insulin receptor was measured by an initial-velocity experiment using a synthetic peptide substrate (Thr-12-Lys) composed of amino acid residues 1143 to 1152 of the human insulin receptor (Goren et al., 1987; White et al., 1988). Thr-12-Lys (Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys) was purchased from Dr. David Coy, Tulane University, New Orleans, Louisiana. The WGA-purified receptor preparation (4 μg of protein) was diluted to 50 μl with 50 mM HEPES (pH 7.4) and a final concentration of 0.1% Triton X-100, 5 mM $MnCl_2$. The basal activity was measured by adding various concentrations of Thr-12-Lys (0.1 mM to 6 mM) to each reaction followed by 50 μM [γ-^{32}P]ATP for 5 min. The stimulated activity was measured by incubating the reaction mixture with insulin (100 nM) for 20 min, followed by 50 μM [γ-^{32}P]ATP for 5 min, and the indicated concentrations of Thr-12-Lys for 5 min. The reaction was stopped after 5 min by adding 20 μl of 1% BSA followed immediately by 50 μl of 10% trichloroacetic acid. The precipitated protein was sedimented by centrifugation, and the supernatant, which contained the phosphorylated peptide, was applied to a 2 × 2 cm piece of phosphocellulose paper (Whatman). The paper was washed with four changes (one liter each) of 75 mM phosphoric acid, and retained radioactivity was measured by Cerenkov counting.

[^{32}P]Phosphorylation of Intact Cells and Immunoprecipitation of Phosphotyrosine-Containing Proteins

Confluent CHO cells in 15 cm dishes were labeled for 2 hr with 0.5 mCi/ml [^{32}P]phosphate (New England Nuclear) as previously described (White et al., 1987). Insulin was added and the incubation was continued at 37°C for the indicated time intervals. The cell monolayers were frozen with liquid nitrogen, thawed, and homogenized immediately at 4°C with 2 ml of a solution containing 50 mM HEPES (pH 7.4), 10 mM sodium pyrophosphate, 100 mM NaF, 4 mM EDTA, 2 mM sodium vanadate, 1 mg/ml aprotinin, and 2 mM phenylmethylsufonyl fluoride (White et al., 1985b). The cells were scraped from the dishes, and insoluble material was sedimented by centrifugation at 50,000 rpm in a Beckman 70.1 Ti rotor for 60 min. The supernatant containing the cytosol fraction was immunoprecipitated with α-PY on Pansorbin (Calbiochem) as described previously; membrane proteins in the particulate fraction were solubilized in extraction solution containing 1% Triton X-100 and then immunoprecipitated with α-PY. Proteins were eluted from washed precipitates with Laemmli sample buffer, reduced with DII (Bio-Rad), and separated by SDS-PAGE on 7.5% resolving polyacrylamide gels (Kasuga et al., 1985). Phosphoproteins were identified by autoradiography, and radioactivity in the gel fragments was quantified by Cerenkov counting or scanning densitometry.

HPLC Separation of Tryptic Phosphopeptides and Identification of Phosphoamino Acids

Tryptic phosphopeptides were prepared and separated with a Waters HPLC system equipped with a wide-pore C_{18} column (Bio-Rad, RP-318) as previously described (White et al., 1987). All of the radioactivity in the tryptic digest was routinely recovered from the reverse-phase HPLC column. The peak that elutes in fraction 95 at 75% acetonitrile is composed of many positively charged peptides that appear to be incomplete tryptic digests and have not been identified.

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