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Insulin Receptor Kinase Domain Autophosphorylation Regulates Receptor Enzymatic Function*

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We have studied a series of insulin receptor molecules in which the 3 tyrosine residues which undergo autophosphorylation in the kinase domain of the β-subunit (Tyr^{1158}, Tyr^{1162}, and Tyr^{1163}) were replaced individually, in pairs, or all together with phenylalanine or serine by *in vitro* mutagenesis. A *single-Phe* replacement at each of these three positions reduced insulin-stimulated autophosphorylation of solubilized receptor by 45–60% of that observed with wild-type receptor. The *double-Phe* replacements showed a 60–70% reduction, and substitution of all 3 tyrosine residues with Phe or Ser reduced insulin-stimulated tyrosine autophosphorylation by >80%. Phosphopeptide mapping each mutant revealed that all remaining tyrosine autophosphorylation sites were phosphorylated normally following insulin stimulation, and no new sites appeared. The *single-Phe* mutants showed insulin-stimulated kinase activity toward a synthetic peptide substrate of 50–75% when compared with wild-type receptor kinase activity. Insulin-stimulated kinase activity was further reduced in the *double-Phe* mutants and barely detectable in the *triple-Phe* mutants. In contrast to the wild-type receptor, all of the mutant receptor kinases showed a significant reduction in activation following *in vitro* insulin-stimulated autophosphorylation. When studied in intact Chinese hamster ovary cells, insulin-stimulated receptor autophosphorylation and tyrosine phosphorylation of the cellular substrate pp185 in the *single-Phe* and *double-Phe* mutants was progressively lower with increased tyrosine replacement and did not exceed the basal levels in the *triple-Phe* mutants. However, all the mutant receptors, including the *triple-Phe* mutant, retained the ability to undergo insulin-stimulated Ser and Thr phosphorylation. Thus, full activation of the insulin receptor tyrosine kinase is dependent on insulin-stimulated Tris phosphorylation of the kinase domain, and the level of autophosphorylation in the kinase domain provides a mechanism for modulating insulin receptor kinase activity following insulin stimulation. By contrast, insulin stimulation of receptor phosphorylation on Ser and Thr residues by cellular serine/threonine kinases can occur despite markedly reduced tyrosine autophosphorylation.

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Over the past 10 years, a considerable body of data has indicated that insulin receptor tyrosine autophosphorylation and kinase activity play essential roles in insulin receptor signal transmission (1–3). However, the exact molecular mechanisms linking the insulin receptor kinase to cellular enzymes and transport processes remain poorly defined, and a few recent reports have indicated that the kinase-inactive insulin receptor or receptors lacking certain autophosphorylation sites may still mediate some biological responses (4–8). This suggests that signal transmission may involve several distinct molecular pathways including the activation of cellular enzymes by direct tyrosine phosphorylation (9, 10), by binding to a tyrosine-phosphorylated substrate like the recently identified IRS-1, via src homology-2 (SH2) or src homology-3 (SH3) domains (11), or by noncovalent association between the insulin receptor itself and other cellular proteins (12, 13). Each of these pathways may depend to a variable degree on the extent of receptor autophosphorylation and the associated conformational changes in the β-subunit and activation of the tyrosine kinase toward substrates (14, 15).

Insulin binding induces a complex cascade of autophosphorylation in the insulin receptor β-subunit in the intact cell and purified receptor preparations (16, 17). Five tyrosine phosphorylation sites have been identified in two regions of the insulin receptor β-subunit (16, 18, 19). Three of these sites,[1] Tyr^{1158}, Tyr^{1162}, and Tyr^{1163}, are clustered in the kinase domain about 130 residues from the ATP binding site (Fig. 1). Two additional sites are in the C terminus of the β-subunit at Tyr^{1328} and Tyr^{1334} (20, 21). Phosphorylation of all 3 tyrosine residues in the kinase domain (tris-phosphorylation) fully activates the kinase *in vitro* (16), whereas the C-terminal phosphorylation sites play a minor role in regulation of the kinase (21, 22).

[1] Two forms of the insulin receptor have been identified representing alternate splice variants of a single gene with or without exon 11 (30, 31). This results in an inframe addition of 36 bases and 12 amino acids to the C-terminal region of the α-subunit. Both forms have been shown to have equivalent kinase activity (53). All experiments were performed with the exon 11 minus form of the receptor. The amino acids are numbered, however, based on the full length sequence including exon 11. For reference to other publications the following conversion table can be used.

Amino acid	Receptor form	
	−Exon 11	+Exon 11
Lys	1018	1030
Tyr	1146	1158
Tyr	1150	1162
Tyr	1151	1163
Tyr	1316	1328
Tyr	1322	1334

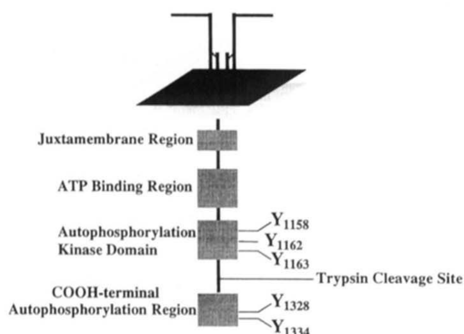

FIG. 1. **Schematic representation of the domains of the insulin receptor β-subunit.** The insulin receptor β-subunit is represented by four regions including the two autophosphorylation regions, one in the C-terminal and the kinase domain, the ATP binding site, and the juxtamembrane region. Mutations involve the replacement of tyrosine residues with phenylalanine or serine in the kinase domain. The two tyrosine autophosphorylation sites in the C-terminal region of the β-subunit can be removed by a trypsin-sensitive cleavage as indicated (20).

Despite the important role of tris-phosphorylation in fully activating the insulin receptor kinase, insulin-stimulated autophosphorylation of the β-subunit in intact hepatoma cells or primary hepatocytes yields primarily bis-phosphorylation of the kinase domain at Tyr^{1158} and either Tyr^{1162} or Tyr^{1163} with only 15–20% of the phosphorylated receptors found to be tris-phosphorylated *in vivo* (16, 19, 23, 24). The difference between *in vivo* and *in vitro* autophosphorylation may be due to regulatory mechanisms *in vivo*, such as dephosphorylation by specific tyrosine phosphatases (25–27). Moreover, the level of phosphorylation in the kinase domain may play an important role in regulating the transmission of the insulin signal by modulating the kinase activity of the receptor.

To study the role of mono-, bis-, and tris-phosphorylation of the insulin receptor kinase domain, we have prepared mutant insulin receptors in which Tyr^{1158}, Tyr^{1162}, and Tyr^{1163} are replaced individually or in combination with phenylalanine or serine. We find that replacement of 1 tyrosine residue with phenylalanine (*single-Phe* replacement) impairs insulin-stimulated kinase activity by more than 50%, and the *double-* and *triple-Phe* replacements reduced activity further, although other phosphorylation sites remain utilized. These results support a hypothesis that insulin receptor kinase activity is dependent on insulin binding and amplified by mono, bis, or tris phosphorylation of the kinase domain and that tris phosphorylation of the kinase domain is essential to fully activating the receptor kinase. However, insulin-stimulated serine/threonine phosphorylation of the β-subunit still occurs in the intact cell in the absence of these tyrosine residues, indicating that autophosphorylation of this region may not be essential for all insulin responses in the intact cell.

MATERIALS AND METHODS

Insulin Receptor Mutagenesis and Expression Vector Construction—Point mutations were introduced into the cDNA of the insulin receptor by oligonucleotide-directed mutagenesis which replaced Tyr^{1158}, Tyr^{1162}, Tyr^{1163}, Tyr$^{1158/1162}$, Tyr$^{1158/1163}$, Tyr$^{1162/1163}$, and Tyr$^{1158/1162/1163}$ with phenylalanine; an additional mutation in which serine replaced all 3 tyrosine residues was also constructed. The template for mutagenesis was prepared by subcloning a 1.9-kilobase *Pst*I-*Pst*I fragment from pSVHIRc (A. Ullrich, Max Planck Institute, Munich) obtained by partial digestion into M13mp18. A uracil-rich template was prepared in *Escherichia coli* strain BW313. The internal *Pst*I site (residues 871–873, based on Ullrich numbering, or residues 883–885, based on Ebina numbering) was altered by site-directed mutagenesis carried out by the method of Kunkel *et al.* (28) to facilitate subsequent cloning steps. The phenylalanine and serine replacements were prepared with the following oligonucleotide primers (DERC Molecular Biology Core Facility, Joslin Diabetes Center), and the mutations were identified by DNA sequencing: F^{1158}, 5′-AGAGACATCTTCGAAACGGAT-3′; F^{1162}, 5′-GAAACGGATTTCTACAGGAAA-3′; F^{1163}, 5′-ACGGATTACTTCCGGAAAGGG-3′; F$^{1158/1162}$, 5′-AGAGACATCTTCGAAACGGATTTCTACCGGAAA-3′; F$^{1158/1163}$, 5′-AGAGACATCTTCGAAACGGATTACTTCCGGAAAGGG-3′; F$^{1162/1163}$, 5′-GAAACGGATTTCTTCCGGAAAGGG-3′; F$^{1158/1162/1163}$, 5′-AGAGACATCTTCGAAACGGATTTCTTCCGGAAAGGG-3′; S$^{1158/1162/1163}$, 5′-AGAGACATCTCTGAAACGGATTCCTCCCGGAAA-3′.

Our expression vector for the wild-type insulin receptor (pSG5HIRc) was constructed by cloning the *Hin*dIII/*Asu*II fragment from pSVHIRc (29) into the polylinker region of pSG5 (Stratagene). The 1.9 kilobase *Pst*I/*Pst*I fragment of the insulin receptor was used to shuttle the mutated cDNA from M13 to pSG5HIRc. Each expression vector was sequenced in the area surrounding the mutation site to confirm the mutation and rule out any other nearby secondary mutation. In addition, the entire 1.9-kilobase insert was sequenced in the IR^{F1158} and IR^{F1158,1162,1163} expression vectors and compared to the corresponding sequence of the wild-type cDNA. In addition to the expected mutations, two differences from the sequence published by Ullrich *et al.* (30) were found in both the wild-type and mutant cDNAs (5′-G*A*C *A*CC-3′ to 5′-G*T*C *T*CC-3′) which change Asp^{861}-Thr^{862} to Val-Ser. This variation was described in the wild-type sequence of Ebina *et al.* (amino acids 873 and 874, based on Ebina numbering) (31). As these changes are present in both the "wild-type" and mutant insulin receptors, they are not responsible for the results of our experiments.

Insulin Receptor Expression in Chinese Hamster Ovary (CHO) Cells and Selection of Clonal Cell Lines—CHO cells were grown in Ham's F12 medium (GIBCO) containing 10% fetal bovine serum (Sigma). The mutant insulin receptors were expressed in CHO cells by co-transfections with the neomycin resistance plasmid pSVEneo and the human insulin receptor expression vector pSGHIRc using calcium phosphate-mediated gene transfer (32). The cells expressing the human insulin receptor constructs were enriched by two rounds of fluorescence-activated cell sorting (DERC Flow Cytometry Core Facility, Joslin Diabetes Center), and clonal cell lines were obtained by limiting dilution (33). Clonal cell lines expressing human insulin receptors were matched for expression level by ^{125}I-insulin (DERC Protein Biochemistry Core Facility, Joslin Diabetes Center) binding, which was analyzed with the LIGAND computer program (34). The presence of the mutant receptor cDNA in the cloned cells was confirmed by direct dideoxy DNA sequencing of the appropriate region of the plasmid DNA amplified by polymerase chain reaction from genomic DNA isolated from these cell lines (35).

In Vitro Autophosphorylation of the Insulin Receptor—Insulin receptors partially purified on wheat germ agglutinin (WGA)[2] agarose were incubated at 23 °C for 30 min in the absence or presence of insulin (10^{-7} M) in 50 mM HEPES, pH 7.4, containing 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 0.1% Triton X-100 (7). The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP (1.0 mM ATP, 3.0 μCi/nmol) to obtain a final concentration of 100 μM ATP. The reaction was terminated after 5 min by the addition of 100 mM Tris, pH 8.0, containing 0.1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium orthovanadate, 2 mM phenylmethylulfonyl fluoride, 1 mg/ml leupeptin, and 10 mg/ml aprotinin. The samples were incubated with monoclonal anti-human insulin receptor antibody (83–14) prepared as previously described (36) and precipitated with Pansorbin (Calbiochem). The proteins were eluted from the complex in Laemmli sample buffer (50 mM Tris, pH 6.9, 10% glycerol, 0.05% bromphenol blue, and 1.0% SDS) containing 100 mM dithiothreitol and separated by SDS-PAGE on a 7% gel (37). The stained and dried gels were autoradiographed, and the ^{32}P incorporated into the insulin receptor β-subunit was quantified by excising the band from the gel and measuring the radioactivity by Cerenkov counting.

Tryptic Phosphopeptide Mapping—WGA-purified insulin receptors were stimulated with insulin (10^{-7} M) and allowed to autophosphorylate for 30 min as described above. The reaction was terminated by the addition of Laemmli sample buffer, and the proteins were sepa-

[2] The abbreviations used are: WGA, wheat germ agglutinin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; Ab, antibody.

rated by SDS-PAGE. Tryptic phosphopeptides were obtained from the insulin receptor β-subunit as previously described (16). The phosphopeptides were separated with a Beckman system Gold HPLC with a RP-318 (Bio-Rad) reverse-phase column. Phosphopeptides applied to the column were eluted at a flow rate of 1.1 ml/min with a mobile phase of 0.05% trifluoroacetic acid in water and an increasing concentration of acetonitrile. Fractions were collected at 1-min intervals, and the amount of radioactivity in each fraction was measured by Cerenkov counting.

In Vitro Kinase Assay—The kinase activity of the insulin receptor was determined by phosphorylation of the peptide substrate *Thr-12-Lys*, which contains the amino acid residues 1154–1165 (Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys) of the human insulin receptor kinase domain (DERC Protein Biochemistry Core Facility, Joslin Diabetes Center). Equal amounts of WGA-purified insulin receptors were diluted into 50 mM HEPES, pH 7.4, containing 10 mM $MgCl_2$ and 10 mM $MnCl_2$. Basal kinase activity was measured during a 5-min interval following the simultaneous addition of *Thr-12-Lys* and [γ-^{32}P]ATP at a final concentration of 600 μM and 100 μM (3 μCi/nmol), respectively. Insulin-stimulated activity was measured in the same way following a 30-min incubation with insulin (10^{-7} M) prior to the addition of Thr-12-Lys peptide and [γ-^{32}P]ATP for 5 min. The effect of insulin-stimulated autophosphorylation on kinase activity was measured by incubating the insulin-stimulated receptors with [γ-^{32}P]ATP for 20 min before adding the Thr-12-Lys peptide for 5 min. The reaction was terminated by the addition of 0.1% bovine serum albumin and 10% trichloroacetic acid, removing the precipitate by centrifugation, and spotting the supernatant on phosphocellulose paper (Whatman). Filters were washed extensively in 75 mM phosphoric acid, rinsed with acetone, dried, and counted for ^{32}P.

Inhibition of insulin-stimulated kinase activity was measured in a kinase assay at various concentrations of *Thr-12-Lys* between 0.1 and 2 mM. Pre-autophosphorylation was achieved by adding [γ-^{32}P]ATP (50 μM ATP final) in the presence of insulin for 15 min. Phosphorylation of *Thr-12-Lys* was allowed to proceed for 5 min at 23 °C at (25 μM ATP, 24 μCi/nmol) and processed as described above.

In Vivo Insulin Receptor and pp185 Tyrosine Phosphorylation—CHO cells expressing wild-type or mutant insulin receptors were grown to confluence on 6-well plates and incubated for 1 min at 37 °C in the absence or presence of insulin (10^{-7} M) in Ham's F-12, pH 7.4, containing 10 mg/ml bovine serum ablumin. The medium was removed, and the reaction was terminated by the addition of 0.3 ml of 2 × Laemmli sample buffer containing 100 mM dithiothreitol and boiling for 1 min. The samples were then sonicated, and proteins were separated on 7% SDS-PAGE gels. Proteins were then transferred to nitrocellulose and blocked overnight at 4 °C in 20 mM Tris, 150 mM NaCl, 3% bovine serum albumin, 0.01% Tween 20, pH 7.4. The blots were then exposed to αPY antibody in blocking solution for 2 h at 23 °C. The antibody solution was removed, and the blots were washed in 20 mM Tris, 150 mM NaCl, 0.01% Tween 20. The bound antibody was detected by incubation with ^{125}I-Protein A for 1 h at 23 °C in blocking buffer (0.1 μCi/ml), the nitrocellulose was washed, and the membrane was autoradiographed. Quantitation was carried out by excising the band of nitrocellulose and counting for ^{125}I.

In Vivo Autophosphorylation and Phosphoamino Acid Analysis—Confluent monolayer of transfected CHO cells in 15-cm plates were labeled for 2 h with 1 mCi of [^{32}P]orthophosphate (Du Pont-New England Nuclear) in 5 ml of phosphate-free RPMI-1640 medium (GIBCO) at 37 °C and 5% CO_2. The labeled cells were incubated in the absence or presence of insulin (10^{-7} M) for 1 min at 37 °C. Cell lysates were prepared in 1% Triton X-100 containing protease and phosphatase inhibitors as described previously (33). Each sample was incubated overnight at 4 °C with a monoclonal anti-insulin receptor antibody 83-14 (36) and immunoprecipitated on Pansorbin (Calbiochem). Immunoprecipitated proteins were separated by SDS-PAGE, stained, destained, dried, and autoradiographed. The phosphoamino acid composition of the labeled receptor was determined following *in situ* tryptic digestion as previously described (38).

RESULTS

Insulin Receptor Expression and Insulin Binding—Wild-type and mutant human insulin receptors were expressed in Chinese hamster ovary (CHO) cells. Cells with high levels of receptor expression (10^6/cell) were isolated by fluorescence-activated cell sorting with a monoclonal anti-insulin receptor antibody (Ab 83–14) specific for an epitope in the α-subunit of the receptor followed by cloning at limiting dilution. When compared to cells expressing the wild-type receptor, insulin binding to the mutant receptors was normal. Scatchard analysis using a two-site model revealed average high and low affinity dissociation constants of 0.6 ± 0.1 nM and 55 ± 11 nM, respectively, for all cell lines. Clonal cell populations of CHO cells used in this study were matched for receptor expression (Table I). Both wild-type and mutant receptors labeled in CHO cells with [^{35}S]methionine and immunoprecipitated with Ab 83–14 displayed mature α- and β-subunits with normal mobility on SDS-PAGE under reducing conditions (data not shown).

In Vitro Autophosphorylation of the Wild-type and Mutant Insulin Receptors—Autophosphorylation of the wild-type and mutant insulin receptors purified from transfected CHO cells by affinity chromatography on wheat germ agglutinin agarose was measured during a 5-min incubation with [γ-^{32}P]ATP (Fig. 2). Insulin stimulated autophosphorylation of the β-subunit of its receptor 8-fold over basal when the wild-type human insulin receptor was isolated from CHO cells and immunoprecipitated with Ab 83–14 (Fig. 2, *A* and *B*). No phosphorylation of the endogenous insulin receptor was observed, since Ab 83–14 is human species specific and does not immunoprecipitate the small number of endogenous rodent insulin receptors in these cells (36). As previously reported (7), replacement of Tyr^{1158} with phenylalanine reduced the basal and insulin-stimulated autophosphorylation by about 50% when compared to wild-type insulin receptor levels (Fig. 2). Although the level of autophosphorylation was decreased, the time course and dose-response of autophosphorylation were unchanged, consistent with the normal insulin binding (Fig. 3, *A* and *B*). The other *single-Phe* mutants, IR^{F1162} and IR^{F1163}, showed decreased insulin-stimulated autophosphorylation similar to that of IR^{F1158} (Fig. 2*B*). However, the basal autophosphorylation of these mutant receptors was higher than the IR^{F1158} and similar to that of the wild-type insulin receptor. On average, *single-Phe* replacements showed a 45–60% reduction in insulin-stimulated autophosphorylation. This was a disproportionately large reduction considering only one of five reported autophosphorylation sites in the β-subunit were replaced.

Insulin-stimulated autophosphorylation of the *double-Phe* mutants ranged between 30 and 50% of control and was

TABLE I

Scatchard analysis of ^{125}I-insulin binding to transfected CHO clonal cell lines

Confluent 24-well plates of each cell line were used in Scatchard cell binding assay. 20,000 cpm of ^{125}I-insulin was added to each well with increasing concentrations of cold insulin in Krebs-Ringer-HEPES binding buffer and incubated 16 h at 4 °C. The binding buffer was removed and the wells washed with ice-cold phosphate-buffered saline. The cells were solubilized in 0.5 ml of 1 N NaOH and counted for ^{125}I.

Cell line	Clone	% of Wild-type insulin receptor expression level	Number of receptors per cell
CHO		3 ± 1	3.3×10^4
IR	CL-1	100	1.0×10^6
IR^{A1018}	3	70 ± 6	0.7×10^6
IR^{F1158}	C-10	91 ± 2	0.9×10^6
IR^{F1162}	A-13	89 ± 4	0.9×10^6
IR^{F1163}	D-8	97 ± 2	1.0×10^6
$IR^{F1158,1162}$	3-9	89 ± 3	0.9×10^6
$IR^{F1158,1162}$	13	95 ± 4	1.0×10^6
$IR^{F1162,1163}$	A-12	107 ± 5	1.1×10^6
$IR^{F1158,1162,1163}$	12	109 ± 2	1.1×10^6
$IR^{S1158,1162,1163}$	8-6	91 ± 3	0.9×10^6

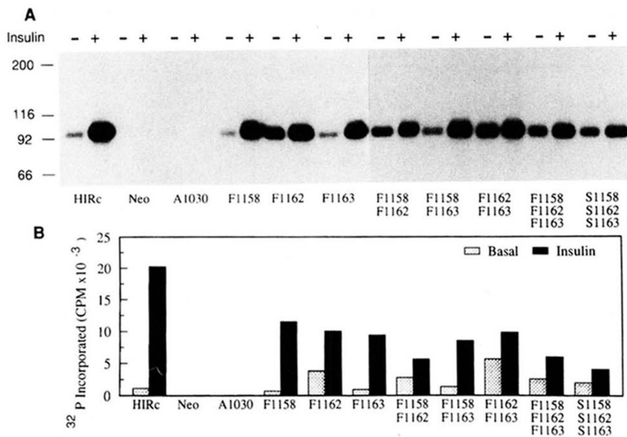

FIG. 2. ***In vitro* autophosphorylation of wheat germ agglutinin-purified insulin receptors.** *Panel A*, equal amounts of WGA-purified wild-type and mutant insulin receptor were treated in the absence or presence of insulin (10^{-7} M) for 30 min at 23 °C. The autophosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP for 5 min at 23 °C. The reaction was terminated, and the receptor was immunoprecipitated with monoclonal anti-human insulin receptor antibody (83–14) and eluted with Laemmli sample buffer. The samples were then separated on 7% SDS-PAGE gels. The gels were then Coomassie-stained, destained, and dried, and the autoradiograph is shown here. *Panel B*, the insulin receptor β-subunit was excised from the gel and counted for [^{32}P]phosphate.

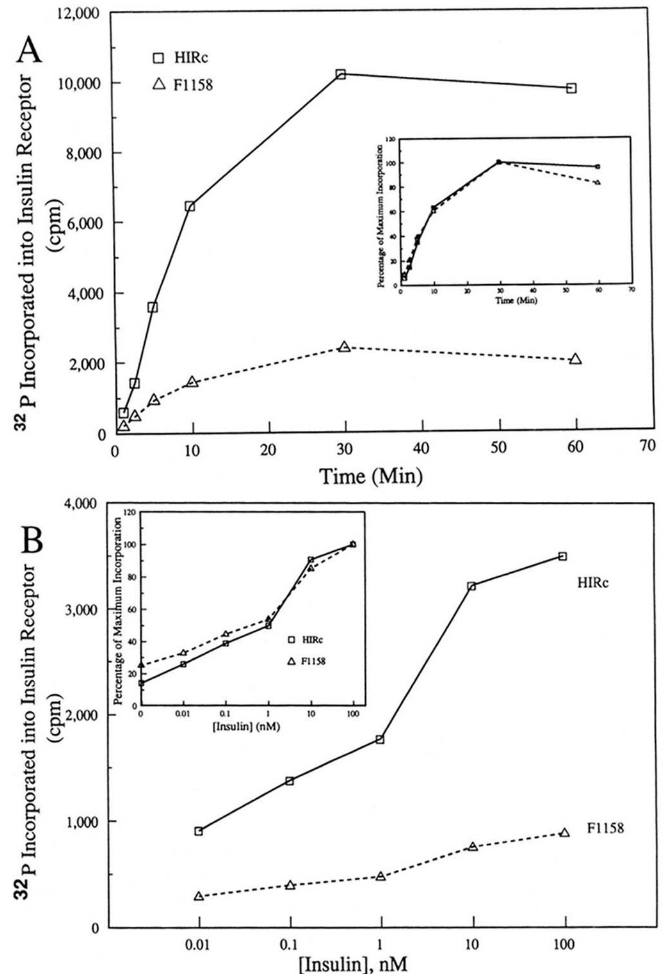

FIG. 3. **Time course and insulin concentration dependence of wild-type and mutant insulin receptor autophosphorylation.** *Panel A*, equal amounts of wild-type and mutant insulin receptors were treated for 1 h at 23 °C in the presence of insulin (10^{-7} M). The autophosphorylation reactions were performed for various times as described under "Materials and Methods." The insulin receptor β-subunit was excised from the gel and counted for [^{32}P]phosphate. The graph represent the counts/min of ^{32}P incorporated into the β subunit of the wild-type or mutant IR^{F1158} as a function of insulin concentration. The *inset* represents the percentage of ^{32}P incorporated by the wild-type and mutant IR^{F1158} insulin receptor at each time point as a function of maximal ^{32}P incorporated. *Panel B*, equal amounts of WGA-purified wild-type and mutant insulin receptors were treated for 1 h in the absence or presence of various concentrations of insulin. The autophosphorylation reaction was performed as described under "Materials and Methods." The insulin receptor β-subunit was excised from the gel and counted for ^{32}P. The *graph* represents the counts/min of ^{32}P incorporated into the β-subunit of the wild-type or mutant IR^{F1158} as a function of insulin concentration. The *inset* represents the percentage of ^{32}P incorporated for wild-type and mutant IR^{F1158} insulin receptor at each insulin concentration as a function of maximal insulin stimulation.

reduced slightly more, on average, than the reduction observed in the *single-Phe* mutants. All three of these mutants, but especially $IR^{F1162,1163}$, exhibited an increased basal autophosphorylation and thus insulin-stimulated autophosphorylation in these mutants is only about 2- to 3-fold when compared to basal (Fig. 2*B*). Autophosphorylation of the *triple-Phe* mutant, $IR^{F1158,1162,1163}$, was also increased in the basal state and stimulated only 2-fold by insulin (Fig. 2, *A* and *B*). Substitution of serine for tyrosine residues, as a potential alternative phosphorylation acceptor sites, did not increase the level of autophosphorylation above that seen in the *triple-Phe* mutant. Thus, serine residues in the position of the normal tyrosine phosphorylation sites were not used as phosphoacceptor sites for the receptor kinase.

Both *triple* mutants were still phosphorylated on tyrosine in response to insulin as they were immunoprecipitated with αPY (data not shown). This residual tyrosine phosphorylation in the *triple-Phe* mutants was localized to the C terminus of the receptor and could be removed by mild trypsin treatment, a treatment which we have previously shown removes a 10-kDa fragment from the C terminus of the β-subunit (20). Thus, the wild-type insulin receptor produced an 85-kDa fragment of the β-subunit which loses the C-terminal phosphorylation sites, but retains the tyrosine phosphorylated kinase domain (Figs. 1 and 4) (16, 20); trypsinization of the *triple-Phe* mutant did not produce a phosphorylated 85-kDa fragment of the β-subunit even though the intact β-subunit was labeled (Fig. 4). The absence of a phosphorylated 85-kDa β-subunit fragment suggests that most of the phosphorylation of the *triple-Phe* mutant occurs in the C terminus.

Tryptic Phosphopeptide Mapping of Insulin Receptor Mutants—To determine which of the tyrosine residues in the mutant insulin receptors were phosphorylated, the WGA-purified mutant insulin receptors were stimulated with insulin and allowed to autophosphorylate for 30 min at 23 °C, and tryptic phosphopeptide maps were derived using reverse-phase HPLC. By 30 min, the insulin receptor autophosphorylation had reached its maximal extent for all the insulin receptor species (Fig. 3*A* and data not shown). The HPLC tryptic phosphopeptide maps of the *triple* mutants confirmed the localization of the remaining phosphorylation to two peaks (pY2 and pY3) (Fig. 5*A*), which we have previously shown to be peptides derived from the C terminus of the insulin receptor β-subunit (39). When analyzing each of the three *double-Phe* mutants, a new peak was seen migrating with a retention time of approximately 94–98 min in our gradient system, as well as the two C-terminal peaks (Fig. 5*B*). This new phosphopeptide peak must correspond to the peptide with monophosphorylation of the kinase domain.

The phosphopeptide maps of the three *single-Phe* mutants,

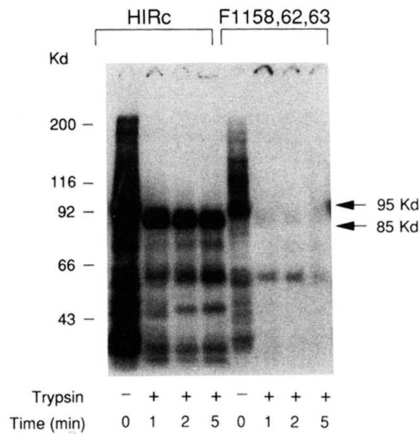

FIG. 4. **Trypsinization of *in vitro* autophosphorylated insulin receptors.** Equal amounts of WGA-purified wild-type and mutant $IR^{F1158,1162,1163}$ insulin receptors were treated in the absence or presence of insulin (10^{-7} M) for 30 min at 23 °C. The autophosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP for 10 min at 23 °C. Sodium vanadate was then added to a final concentration of 400 μM, and 0.5 mg of trypsin was added at 23 °C for 0, 1, 2, or 5 min prior to the addition of Laemmli sample buffer. The samples were then separated on a 7% SDS-PAGE gel. The gel was then Coomassie-stained, destained, dried, and autoradiographed.

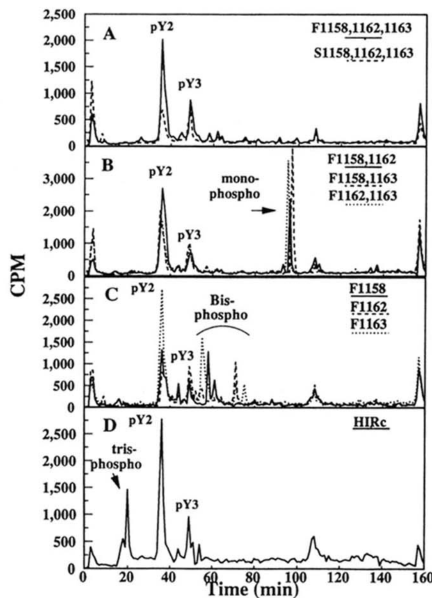

FIG. 5. **Reverse-phase HPLC analysis of tryptic phosphopeptides derived from autophosphorylated WGA-purified insulin receptors.** The insulin receptor *triple* mutants (*panel A*), *double-Phe* mutants (*panel B*), *single-Phe* mutants (*panel C*), and wild-type (*panel D*), partially purified on WGA were stimulated with insulin (10^{-7} M) and allowed to autophosphorylate for 30 min at 23 °C in the presence of [γ-^{32}P]ATP. Gel fragments containing the autophosphorylated insulin receptor β-subunit were excised and digested with 0.2 mg/ml trypsin for 18 h, and the resulting peptides were resolved by reverse-phase HPLC as described under "Materials and Methods."

which allow either mono- or bis-phosphorylation of the kinase domain, were slightly more complex. Each of the these maps included one or two new peaks with a retention time of between 55 and 75 min in addition to the two C-terminal peaks, but no mono-phosphorylated kinase domain peak was observed at 94–98 min (Fig. 5*C*). The peaks at 55 and 75 min presumably represent the various bis-phosphorylated forms of the peptide derived from the kinase domain.

The phosphopeptide maps of these insulin receptor mutants were compared to the phosphopeptide map of the wild-type receptor. The HPLC profile of the tryptic digest of the wild-type insulin receptor included two peaks migrating before the two C-terminal peaks with a retention time of 16 and 19 min (Fig. 5*D*). These latter two peaks have previously been identified as the tris-phosphorylated kinase domain (39). All of the new peaks generated in the *single-Phe* and *double-Phe* mutants were immunoprecipitable by an anti-peptide antibody raised to the Thr-12-Lys peptide which includes all 3 of the tyrosine residues in the kinase domain. Thus, it appears that all of the tyrosine residues remaining in the kinase domain of the mutant insulin receptors can be phosphorylated.

Activation of the Insulin Receptor Kinase—Although insulin stimulates the insulin receptor kinase activity, the major amplification of kinase activity as assessed *in vitro* assays with exogenous substrates and synthetic peptides, follows autophosphorylation of the insulin receptor kinase domain (16). As shown in Fig. 6, before insulin stimulation, both the wild-type and mutant insulin receptors exhibited a low level of kinase activity toward the synthetic peptide *Thr-12-Lys* (Fig. 6). In the absence of autophosphorylation of the insulin receptor, insulin (10^{-7} M) stimulated this kinase activity 4-fold in the wild-type receptor. However, autophosphorylation for 20 min in the presence of insulin and 1 mM ATP resulted in an additional tripling of substrate phosphorylation, producing an overall 12-fold stimulation of kinase activity. The amplified kinase activity measured after autophosphorylation is thought to result from tris-phosphorylation of the kinase domain (Fig. 5*D*) (16, 17). Thus, insulin-stimulated *in vitro* kinase activity is activated by insulin binding and strongly modulated by the level of autophosphorylation of the kinase domain with tris-phosphorylation of this region resulting in maximal activation of the insulin receptor kinase.

All three of the *single-Phe* receptor mutants retained between 48 and 78% of the insulin-stimulated kinase activity toward exogenous peptide when compared to the wild-type insulin receptor. Interestingly, autophosphorylation of these *single-Phe* mutants for 20 min with insulin and ATP produced little further increase in the kinase activity. Thus, these mutant receptor molecules largely retain their capacity for insulin-stimulated activation, but have lost most of their ability to increase their kinase activity due to autophosphorylation. This is consistent with the conclusion that maximal

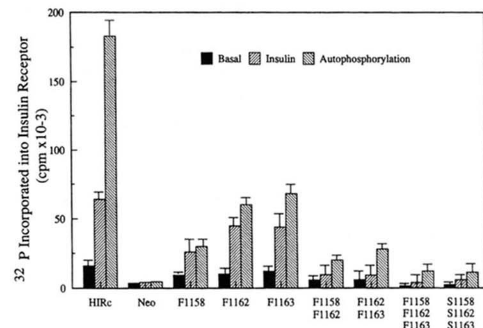

FIG. 6. **Activation of the wild-type and mutant insulin receptor kinases by insulin and autophosphorylation.** Equal amounts of WGA-purified wild-type and mutant insulin receptors were untreated (*Basal*), treated with insulin (10^{-7} M) (*Insulin*), or treated with insulin and allowed to autophosphorylate (*Autophosphorylation*) prior to incubation with Thr-12-Lys peptide (600 μM) for 5 min at 23 °C as described under "Materials and Methods." Background counts (3000 cpm) in the absence of substrate were subtracted from each assay point. The values are the averages of three experiments with the *error bar* representing standard error of the mean.

kinase activation requires tris-phosphorylation of the kinase domain, although bis-phosphorylation may lead to a slight enhancement of the kinase activity.

Insulin-stimulated kinase activity of each of the three *double-Phe* mutant receptors was reduced on average to about 20% of the wild-type level. The insulin-stimulated kinase activity was not statistically above the basal during our kinase assay and only slightly higher than that of the endogenous rodent receptor (Fig. 6). Furthermore, the insulin-stimulated kinase activity was far below that of the *single-Phe* mutants and the wild-type receptor, suggesting that at least two phosphorylation sites are necessary for the majority of insulin-stimulated kinase activity. Autophosphorylation of the *double-Phe* mutants, which allows only mono-phosphorylation of the kinase domain, increased the kinase activity slightly, but this was still far below the normal level of activation seen with the wild-type insulin receptor (*i.e.* tris-phosphorylation) and also below the levels of activation seen with the *single-Phe* mutants (*i.e.* bis-phosphorylation). The two *triple* mutants were further reduced in activity with neither exhibiting any significant increase in kinase activity during autophosphorylation. Thus, full kinase activity requires tris-phosphorylation, and kinase activation is progressively lost as these three autophosphorylation sites are replaced.

Inhibition of the Insulin Receptor Tyrosine Kinase Activity by High Concentrations of Exogenous Substrate—We have previously shown that the substrate peptide *Thr-12-Lys* at millimolar concentrations inhibits insulin-stimulated tris-phosphorylation of the β-subunit and blocks the full activation of the insulin receptor kinase (16). Thus, concentration dependence curves of substrate phosphorylation obtained before the receptor is fully phosphorylated are biphasic reaching a peak at 0.6 mM peptide then falling off with half-maximal inhibition occurring at approximately 1 mM peptide (16). In the absence of insulin stimulation, the V_{max} for phosphorylation of the *Thr-12-Lys* by wild-type receptors was about 0.1 pmol/min (Fig. 7*A*). After 15 min of insulin-stimulated autophosphorylation, which yields about 75% of the maximum level of autophosphorylation (Fig. 3*A*), insulin receptor kinase activity was stimulated more than 10-fold during assays with *Thr-12-Lys*, provided the concentration of substrate was below 0.75 mM (Fig. 7*A*). However, above this concentration, there was a significant inhibition of the activity, which we attributed to inhibition of autophosphorylation by the excess peptide concentrations during the kinase assay (Fig. 7*A*); allowing additional autophosphorylation to occur before adding the peptide completely eliminates the biphasic curve (16).

The substrate concentration curves for insulin-stimulated phosphorylation by all of the mutants were lower than that of the wild-type receptor, but above that of the endogenous rodent receptor (Fig. 7, *B–E*). The kinase activity of all three *single-Phe* mutants was reduced 60 to 70% compared to the wild-type receptor (an example for IR^{F1158} is shown in Fig. 7*C*); with these mutants there was also a small degree of inhibition at high substrate concentration. A more marked reduction in insulin-stimulated kinase activity was seen with the *double-Phe* mutant ($IR^{F1158, 1162}$ in Fig. 7*D*), and the activity of the *triple-Phe* mutants was undetectable above the level of the endogenous receptor (Fig. 7, compare *E* and *B*). Thus, tris-phosphorylation of the insulin receptor kinase domain to undergo leads to a higher level of kinase activation than either mono- or bis-phosphorylation. Furthermore, only insulin receptors which undergo either tris- or bis-phosphorylation exhibit inhibition at high substrate concentrations, indicating some level of activation of these receptor constructs due to autophosphorylation.

In Vivo Tyrosine Phosphorylation of the Wild-type and Mutant Insulin Receptors and pp185 in CHO Cells—CHO cells expressing equal amounts of wild-type and mutant human insulin receptors (Table I) were incubated in the absence or presence of insulin (10^{-7} M) for 1 min at 37 °C. In the absence of insulin stimulation, the major phosphotyrosine-containing proteins in all cell lines had molecular weights of 120,000 and 70,000; tyrosine phosphorylation of the insulin receptor β-subunit and its endogenous substrate, pp185, was either at or only slightly above the level of detection by αPY antibody blotting (Fig. 8*A*). Stimulation of CHO-HIRc cells by insulin lead to an 8-fold increase in insulin receptor autophosphorylation and a dramatic increase in tyrosine phosphorylation of pp185 (Fig. 8, *B* and *C*). Insulin stimulation of all three *single-Phe* mutants lead to an increase in insulin receptor autophos-

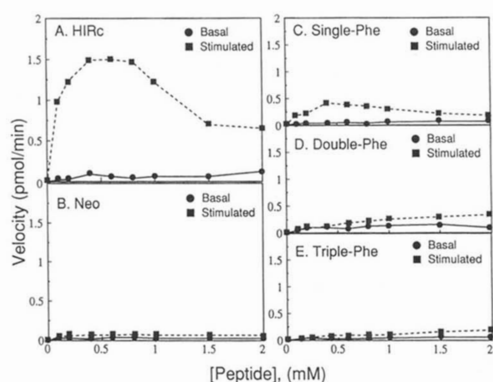

FIG. 7. **Inhibition of wild-type and mutant insulin receptor kinase activity with peptide substrate.** Equal amounts of WGA-purified wild-type (*panel A*) and mutant insulin receptors (*panel C*, IR^{F1158}; *panel D*, $IR^{F11581162}$; *panel E* $IR^{F1158, 1162, 1163}$) were incubated in the presence of insulin (10^{-7} M) and [γ-^{32}P]ATP (50 mM ATP, 24 μCi/nmol) for 15 min at 23 °C. The substrate kinase assay was initiated by the addition of various concentrations of peptide (Thr-12-Lys) for 5 min at 23 °C. The reaction was terminated as described under "Materials and Methods." Background counts (2200 cpm) in the absence of substrate were subtracted from each assay point.

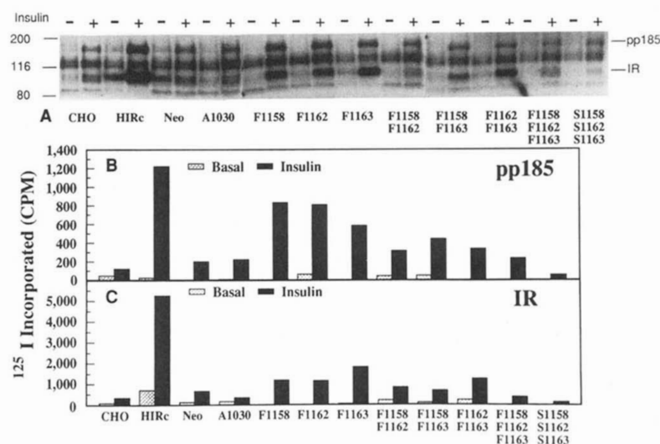

FIG. 8. ***In vivo* insulin stimulation of insulin receptor autophosphorylation and tyrosine phosphorylation of pp185.** Tyrosine phosphorylation of the insulin receptor β-subunit and pp185 was measured in CHO cells expressing the wild-type and mutant insulin receptors by αPY antibody blotting using ^{125}I-Protein A following a 1-min insulin (10^{-7} M) stimulation at 37 °C. Cells were extracted, and proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected by αPY antibody blotting as described under "Materials and Methods." The autoradiograph of such an experiment is seen in *panel A*. Tyrosine phosphorylation of the insulin receptor β-subunit (*panel C*) and pp185 (*panel B*) was quantified by scanning laser densitometry of the autoradiographs using a Molecular Dynamics computer densitometer.

phorylation; however, the level of phosphorylation was reduced to about 30% of that seen with the wild-type receptor. Insulin stimulation of tyrosine phosphorylation of pp185 was also reduced in these cells, but to a lesser extent ranging from 55–70% of the level in CHO-HIRc cells. With the double-Phe mutants, insulin-stimulated tyrosine phosphorylation of the receptor β-subunit and pp185 was reduced on average to 15% and 30%, respectively, when compared to levels in CHO-HIRc cells. Insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit and pp185 in the *triple* mutants was similar to that seen in the untransfected parental (CHO) cell line, the mock-transfected (CHO-Neo) cell line, and cells expressing the kinase-inactive receptor (CHO-IR^{A1030}). Thus, progressive elimination of tyrosine autophosphorylation sites in the kinase domain leads to the progressive reduction in receptor autophosphorylation in intact cells, and this is paralleled by the progressive reduction of insulin-stimulated pp185 phosphorylation. However, in the *single* and *double* mutants, the reduction in pp185 phosphorylation is somewhat less than that in the receptor itself suggesting that some amplification of the signal still persists.

In Vivo Phosphorylation of the Wild-type and Mutant Insulin Receptor Expressed in CHO Cells—To assess serine and threonine as well as tyrosine phosphorylation, CHO cells expressing equal numbers of wild-type and mutant human insulin receptors were labeled with [^{32}P]orthophosphate for 2 h and then incubated in the absence or presence of insulin (10^{-7} M) for 1 min at 37 °C. Without insulin stimulation, all of the receptors displayed nearly equal basal phosphorylation as assessed by immunoprecipitation with the human specific anti-insulin receptor antibody 83–14 (Fig. 9). As we have previously demonstrated, this basal phosphorylation in intact cells is due entirely to serine and threonine phosphorylation (40); thus, these receptors were not immunoprecipitated (data not shown) or immunostained with αPY antibody (Fig. 8). Interestingly, the basal phosphorylation of the two *double-Phe* mutants was different, being significantly lower for the $IR^{F1158,1162}$ and higher for the $IR^{F1162,1163}$. This increase for the $IR^{F1162,1163}$ has been reported previously by Debant and coworkers (6).

As previously reported, when compared to the basal level of phosphorylation, insulin stimulated phosphorylation of the wild-type insulin receptor approximately 4-fold, whereas the ATP binding site, kinase-inactive receptor mutant (IR^{A1030}), displayed no stimulation (Fig. 9, *A* and *B*). The endogenous rodent receptor was not detected in CHO-Neo cells in these experiments due to use of the human specific anti-insulin receptor antibody (Fig. 9, *A* and *B*). All of the mutant insulin receptors retained approximately a 2-fold insulin-stimulated phosphorylation based on immunoprecipitation of the total receptor pool with anti-receptor antibody 83–14. The *double-Phe* mutants were again distinguished by the fact that insulin-stimulated phosphorylation of the $IR^{F1158,1162}$ was extremely low, whereas the $IR^{F1162,1163}$ was nearly equal to the wild-type receptor. However, insulin stimulation of the $IR^{F1162,1163}$ was still 2-fold owing to its high basal level of phosphorylation.

Insulin stimulation of all of the *single-* and *double-Phe* mutants occurred to some extent on tyrosine residues as indicated by αPY antibody blotting (Fig. 8, *A* and *C*), but the level of labeling was substantially reduced when compared to the wild-type insulin receptor. Insulin stimulated phosphorylation of the *triple-Phe* mutant in CHO cells, but this phosphorylated receptor was not immunoprecipitated with anti-phosphotyrosine antibody (data not shown), indicating that insulin stimulated primarily Ser/Thr phosphorylation in this mutant. To confirm this point, phosphoamino acid analysis was performed with the wild-type and mutant receptor. Phosphoamino acid analysis revealed that basal phosphorylation of both the wild-type insulin receptor and mutant $IR^{F1158,1162,1163}$ occurred on serine and threonine (Fig. 10). Insulin-stimulated phosphorylation of all three phosphoacceptor amino acids in the wild-type insulin receptor, but only phosphoserine and phosphothreonine levels were increased by insulin in the $IR^{F1158,1162,1163}$ (Fig. 8). Insulin receptor mutants with *single-* and *double-Phe* mutations showed an increase in phosphoserine and phosphothreonine, as well as an increase in phosphotyrosine as predicted (data not shown).

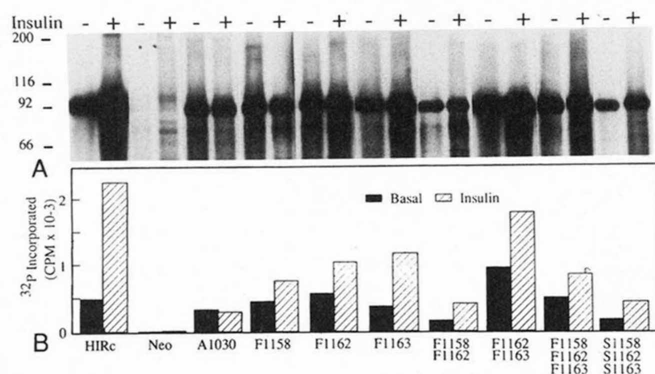

FIG. 9. ***In vivo*** **^{32}P-labeling of clonal CHO cells expressing wild-type and mutant insulin receptor.** Two confluent 15-cm plates of each clonal cell line were labeled with [^{32}P]orthophosphate *in vivo* as described under "Materials and Methods." Cells were incubated in the absence or presence of insulin (10^{-7} M) for 1 min at 37 °C. The cell extracts were immunoprecipitated with monoclonal anti-insulin receptor antibody 83–14 (*panel A*). The insulin receptors were eluted from antibody/Pansorbin complex with boiling Laemmli sample buffer and separated on 7% SDS-PAGE gels. The gels were Coomassie-stained, destained, and dried, and the autoradiograph is shown. *Panel B*, the insulin receptor β-subunit was excised from the gel and counted for [^{32}P]phosphate. This is representative of three experiments.

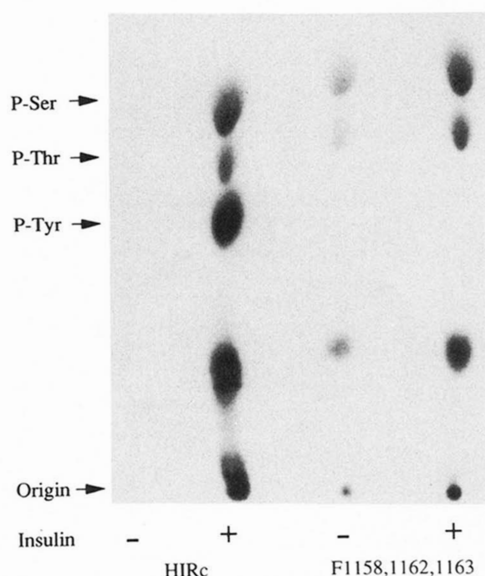

FIG. 10. **Phosphoamino acid analysis of *in vivo* ^{32}P-labeled insulin receptors.** The wild-type and mutant $IR^{F1158,1162,1163}$ insulin receptors were ^{32}P-labeled and immunoprecipitated with anti-insulin receptor antibody as described for Fig. 9. The β-subunit was excised, trypsinized, hydrolyzed in 6 N HCl for 1.5 h at 110 °C for phosphoamino acid analysis as described under "Materials and Methods." The samples were labeled in the absence and presence of insulin (10^{-7}) for 1 min at 37 °C. The *markers* indicate the migration of the ninhydrin-stained phosphoamino acid standards.

DISCUSSION

Immediately following insulin binding to the α-subunit of the insulin receptor, the β-subunit of the receptor undergoes autophosphorylation on 5 tyrosine residues, including Tyr^{1158}, Tyr^{1162}, and Tyr^{1163} in the kinase domain and Tyr^{1328} and Tyr^{1334} in the C terminus (16, 18, 19, 23). We have previously suggested that autophosphorylation occurs in the kinase domain first as an intramolecular cascade and is followed by phosphorylation in the C terminus (16, 41). Our studies and others also suggested that this tris-phosphorylation of the kinase domain is required to fully activate the tyrosine kinase (7, 16, 17), whereas the C-terminal sites do not play a detectable role (21), although this latter point is controversial (22, 42). The present study further emphasizes the role of kinase domain autophosphorylation in receptor activation. Thus, replacement of any single tyrosine residue in the kinase domain with phenylalanine (*single-Phe*) reduces the kinase activity that occurs after insulin-stimulated autophosphorylation by more than 50% and there is a nearly complete loss of insulin-stimulated activation when all three phosphorylation sites are replaced. Based on these results, we conclude that insulin stimulates the receptor kinase and the kinase activity is modulated by the relative level of autophosphorylation in the kinase regulatory domain. In the intact cell, regulation of autophosphorylation may also be modulated by regulatory proteins (43) or specific phosphatases (25–27) which might influence insulin-stimulated autophosphorylation, thus providing a fine level of regulation of the initial step of insulin signal transmission.

We originally proposed that the autophosphorylation cascade in the β-subunit proceeds in an ordered sequence beginning with Tyr^{1158} (16). This conclusion was based on the fact that the anti-phosphotyrosine (αPY) antibody inhibits the autophosphorylation cascade after bis-phosphorylation of the kinase domain at Tyr^{1158} and either Tyr^{1162} or Tyr^{1163} (16, 21). However, replacement of Tyr^{1158} with phenylalanine does not completely block autophosphorylation of the remaining sites in the kinase domain or in the C terminus (7, 44). Moreover, the IR^{F1158} undergoes autophosphorylation at the same rate as the wild-type insulin receptor, even though the maximal level of phosphorylation is reduced more than 50%. Similar results were seen with *single-Phe* mutants IR^{F1162} and IR^{F1163}. Phosphopeptide mapping indicates that in all of the mutant insulin receptors the remaining tyrosine autophosphorylation sites can be phosphorylated after insulin stimulation. Thus, the present study indicates that the autophosphorylation cascade is affected by mutation at any of the 3 tyrosines to almost the same extent and suggests that the insulin-stimulated autophosphorylation cascade may not proceed by an obligatory ordered reaction of the tyrosine residues in the kinase domain. Nevertheless, kinase domain phosphorylation appears to be preferred over C-terminal phosphorylation in the wild-type receptors, and tris-phosphorylation of the kinase domain is required for full kinase activation.

As noted above, substitution of any one of the tyrosine residues in the kinase domain reduces insulin-stimulated kinase activity more than 50%. This decrease in kinase activity of the *single-Phe* mutants is largely due to the lack of full receptor activation that occurs after complete autophosphorylation of the wild-type receptor. However, all *single-Phe* mutants retain a significant level of insulin-stimulated kinase activity. These results suggest that the kinase activation which occurs during our *in vitro* assays can be divided into two phases. An initial activation which is dependent on insulin binding but independent of autophosphorylation and a second phase which amplifies the kinase activity by autophosphorylation of the kinase domain, thus regulating the overall level of kinase activity. Complete receptor kinase activation occurs after full insulin-stimulated autophosphorylation of the insulin receptor and is detected to the greatest extent with the wild-type insulin receptor. Based on previous experiments by us and others (2, 7, 16), complete kinase activation occurs only after extensive autophosphorylation of the receptor resulting in tris-phosphorylation of the kinase domain; however, bis- or mono-phosphorylation of the kinase domain may partially support and modulate kinase activity. However, since none of the *single-*, *double-*, and *triple-Phe* mutants can reach the full tris-phosphorylation, they are unable to show full kinase activation. These results provide the most direct support for the previous conclusion that tris-phosphorylation of the kinase domain is necessary for complete activation of the insulin receptor kinase (7, 16).

Our results are discordant with those of Zhang *et al.* (44) who reported that the kinase activity of the IR^{F1158} was stimulated normally by insulin. Their assays were performed using the wild-type insulin receptor and the IR^{F1158} immunopurified from transfected 3T3 cells using an immobilized monoclonal antibody (44). The exact explanation for this difference is unknown. It is possible that the antibody used (2G7) prevents a conformational change in the wild-type insulin receptor that ordinarily occurs after tris-phosphorylation; alternatively, the antibody may facilitate a conformational change in the IR^{F1158} that ordinarily requires tris-phosphorylation. However, the data of the current study with the other *single-Phe* mutants, as well as previous data from our laboratory on IR^{F1158}, are consistent with the conclusion that full kinase activity does not occur in the absence of tris-phosphorylation in the kinase domain (2). Direct comparison of the assay conditions may reveal important information regarding the mechanisms of receptor activation.

In intact cells, bis-phosphorylation of the kinase domain is the predominant phosphorylation state of the insulin receptor β-subunit following insulin stimulation (16, 19, 23, 45). The low level of tris-phosphorylation may be due in part to the action of phosphotyrosine phosphatases (25–27), a regulatory effect of the cell membrane or interaction of the receptor with other molecules which modify the autophosphorylation reaction in the intact cell (43). Based on the current results, the bis-phosphorylated receptor does not exhibit a fully activated kinase activity in the intact cell.

In vivo basal phosphorylation of the insulin receptor occurs on serine and threonine residues, and insulin-stimulated receptor phosphorylation is due to increases in phosphoserine and phosphothreonine and tyrosine autophosphorylation (40). We find that the basal phosphorylation state of the mutant insulin receptors expressed in CHO cells is relatively constant and is also due exclusively to phosphoserine and phosphothreonine. Interestingly, insulin stimulates serine and threonine phosphorylation of the *single-Phe* and *double-Phe* mutants in a manner similar to that for the wild-type receptor, despite the decreased level of tyrosine autophosphorylation. This is consistent with reports by others for the $IR^{F1162,1163}$ (2, 6), although it has been reported that the insulin-stimulated phosphorylation of IR^{F1162} *in vivo* is due to tyrosine phosphorylation only (41).

Insulin stimulation of the *triple-Phe* mutant phosphorylation in intact CHO cells is markedly reduced, but significantly above that found in the CHO-Neo cells. This residual phosphorylation is due entirely to increases in phosphoserine and phosphothreonine, consistent with the findings that the *triple-Phe* insulin receptor mutant shows little or no tyrosine kinase activity following insulin stimulation. It is possible

that this mutant receptor is capable of transmitting some type of biological signal independent of tyrosine kinase activity or requires only a very low level of tyrosine kinase activity (due either to the endogenous rodent insulin receptors or the low activity of the mutant) to stimulate Ser/Thr phosphorylation. Alternatively, insulin binding alone, or in combination with ATP binding, induces some conformational change in the receptor which allows Ser/Thr phosphorylation by cellular kinases (46). Our results are similar to those of Murakami and Rosen (47), but are in contrast to those of Sung and coworkers who have reported *in vivo* tyrosine autophosphorylation of the $IR^{F1158,1162,1163}$ equal to that of the wild-type insulin receptor using anti-phosphotyrosine antibody immunobloting when this receptor is expressed in HTC cells (4). The reason for the disparity in these studies is not known, but differences due to the host cell line or specific receptor constructions used (plus or minus exon 11) are possible explanations. In addition, overexpression of the human insulin receptor can lead to the formation of hybrid receptors between the human insulin receptor half and either the endogenous rodent insulin receptor half or the endogenous rodent insulin-like growth factor-1 (IGF-I) receptor half (48–50). The effects *in vitro* of insulin and IGF-I receptor hybrids on autophosphorylation and kinase activity are only now beginning to be explored (51, 52).

In summary, the data of the current study, along with other studies, indicate that the activation of the insulin receptor kinase involves two phases. Activation of the insulin receptor kinase is dependent on insulin binding and is further amplified by the phosphorylation state of the 3 tyrosine residues in the kinase domain. The increase in phosphorylation state of the kinase domain from the dephospho-form through mono-, bis-, and finally tris-phosphorylation leads to an increase in kinase activity reaching maximal activation upon tris-phosphorylation of the kinase domain. Thus, phosphorylation of the tyrosine residues of the kinase domain regulates the enzymatic function of the insulin receptor. In addition, insulin treatment increases insulin receptor Ser/Thr phosphorylation without kinase activation via an unknown mechanism. Further studies will elucidate the role of the insulin receptor kinase domain autophosphorylation in signaling insulin stimulation of cellular bioactivity.

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