Mutations in the Juxtamembrane Region of the Insulin Receptor Impair Activation of Phosphatidylinositol 3-Kinase by Insulin

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CHO/$IR_{F960/T962}$ cells express a mutant human insulin receptor in which Tyr960 and Ser962 in the juxtamembrane region of the receptor's β-subunit are replaced by Phe and Thr, respectively. The mutant insulin receptor undergoes autophosphorylation normally in response to insulin; however, insulin fails to stimulate thymidine incorporation into DNA, glycogen synthesis, and tyrosyl phosphorylation of an endogenous substrate pp185 in these cells. Another putative substrate of the insulin receptor tyrosine kinase is phosphatidylinositol 3-kinase (PtdIns 3-kinase). We have previously shown that PtdIns 3-kinase activity in Chinese hamster ovary cells expressing the wild-type human insulin receptor (CHO/IR) increases in both antiphosphotyrosine [anti-Tyr(P)] immunoprecipitates and intact cells in response to insulin. In the present study a new technique (detection of the 85-kDa subunit of PtdIns 3-kinase using [^{32}P]phosphorylated polyoma virus middle T-antigen as probe) is used to monitor the PtdIns 3-kinase protein. The 85-kDa subunit of PtdIns 3-kinase is precipitated by anti-Tyr(P) antibodies from insulin-stimulated CHO/IR cells, but markedly less protein is precipitated from CHO/$IR_{F960/T962}$ cells. The amount of PtdIns 3-kinase activity in the immunoprecipitates was also reduced in the CHO/$IR_{F960/T962}$ cells compared to CHO/IR cells. In intact CHO/$IR_{F960/T962}$ cells, insulin failed to stimulate phosphate incorporation into one of the products of activated PtdIns 3-kinase, phosphatidylinositol-3,4-bisphosphate[PtdIns(3,4)P_2], whereas it caused a 12-fold increase in CHO/IR cells. In contrast, phosphate incorporation into another product, phosphatidylinositol trisphosphate [PtdInsP_3], was only partially depressed in the CHO/$IR_{F960/T962}$ cells. The data indicate that disruption of the juxtamembrane region of the insulin receptor impairs its ability to modulate PtdIns 3-kinase activity and suggest that PtdIns 3-kinase may play an important role in insulin signaling. The results further suggest that the levels of PtdIns(3,4)P_2 and PtdInsP_3 can be differentially regulated in the intact cell, and that production of the former may be important for some of the biological actions of insulin. (Molecular Endocrinology 5: 769–777, 1991)

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INTRODUCTION

The cellular targets of the insulin receptor have remained elusive despite considerable research. Recently, we (1) and others (2) have described the activation of phosphatidylinositol 3-kinase by insulin. The enzymatic activity could be immunoprecipitated with anti-Tyr(P) antibodies within a minute after stimulation of intact cells with insulin (1, 2), and the reaction prod-

ucts, PtdIns(3, 4)P_2[1] and PtdInsP_3 were elevated in intact cells with a similar time course (1). In addition, a fraction of the PtdIns 3-kinase could be immunoprecipitated with an antiinsulin receptor antibody after insulin addition, indicating that this enzyme is a direct substrate of the insulin receptor (1).

Phosphatidylinositol 3-kinase has been implicated as playing a role in the signal transduction pathways of a variety of growth factors and oncogenes (3). This enzyme phosphorylates the D-3 position of the inositol ring of PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2, generating PtdIns(3)P, PtdIns(3,4)P_2, and PtdInsP_3, respectively (4–6). The last two products have been shown to increase in intact cells in response to PDGF (5), colony stimulating factor-1 (7), and insulin (1) and in cells transformed by polyoma virus (8, 9) and the *abl* oncogene (10), whereas the levels of PtdIns(3)P are not significantly altered. Furthermore, several mutants of the PDGF receptor, polyoma middle T and *abl*, which fail to promote growth or transformation, respectively, also fail to activate PtdIns 3-kinase (10–14). However, the manner in which PtdIns 3-kinase and its lipid products may contribute to signal transduction is still not clear.

The PtdIns 3-kinase purified from rat liver is a heterodimer of 85- and 110-kDa subunits (6). The 85-kDa subunit specifically associates with the middle T-antigen of polyoma virus *in vitro* (6). This same protein associates with the polyoma middle T/pp60$^{c\text{-}src}$ complex in middle T-transformed cells and with the PDGF receptor in PDGF-stimulated cells (15–18). In both cases the 85-kDa protein becomes phosphorylated on tyrosine residues (15, 16). These findings suggest that PtdIns 3-kinase is a substrate for the receptor and nonreceptor families of tyrosine kinases and that it may play a role in their ability to modulate cell signaling.

Recently, a cell line [Chinese hamster ovary (CHO)/$IR_{F960/T962}$] expressing a double mutant of the human insulin receptor has been extensively characterized with respect to signaling (19). The Tyr960 and Ser962 residues of the receptor's cytoplasmic juxtamembrane region have been replaced by Phe and Thr, respectively. Insulin receptor molecules containing these two point mutations are similar to wild-type receptors in regard to insulin-stimulated autophosphorylation *in vivo* and kinase activity toward exogenous substrates *in vitro* (19). However, in intact cells expressing the mutant receptor, the ability of insulin to stimulate glycogen synthesis, amino acid uptake, thymidine incorporation into DNA, and phosphorylation of a putative endogenous substrate, pp185, are all impaired (19). Thus, the juxtamembrane region of a functional insulin receptor kinase appears to be important for tyrosyl phosphorylation of cellular proteins and signal transduction by the insulin receptor.

In this study CHO cells transfected with the wild-type insulin receptor (CHO/IR) or the mutant receptor molecule (CHO/$IR_{F960/T962}$) were used to investigate the activation of PtdIns 3-kinase by the insulin receptor and its potential importance in insulin signalling. Here we show that 85-kDa subunit and PtdIns 3-kinase activities were reduced 60–80% in anti-Tyr(P) immunoprecipitates from insulin-stimulated CHO/$IR_{F960/T962}$ cells compared to those in CHO/IR cells. In addition, in intact CHO/$IR_{F960/T962}$ cells insulin failed to increase the level of [^{32}P]phosphate incorporated into PtdIns(3,4)P_2. Phosphate incorporation into PtdInsP_3 was partially diminished in the mutant cells. These findings suggest that the juxtamembrane region of the insulin receptor plays an important role in the regulation and activation of PtdIns 3-kinase activity by the activated insulin receptor.

RESULTS

PtdIns 3-Kinase Activity in Antiphosphotyrosine Immunoprecipitates of Insulin-Stimulated CHO/IR, CHO/$IR_{F960/T962}$ and CHO/NEO Cells

Insulin increases immunoprecipitable PtdIns 3-kinase activity in CHO cells expressing the wild-type human insulin receptor (CHO/IR) (1, 2). To investigate whether the juxtamembrane region of the insulin receptor is required for this effect, PtdIns 3-kinase was assayed in anti-Tyr(P) immunoprecipitates from CHO/$IR_{F960/T962}$ cells.

As shown in Fig. 1, whereas treatment of CHO/IR cells with insulin resulted in an increase in anti-Tyr(P)-immunoprecipitable PtdIns 3-kinase, this effect was markedly diminished in CHO/$IR_{F960/T962}$ cells. The anti-Tyr(P)-immunoprecipitable PtdIns 3-kinase activity of insulin-treated CHO/$IR_{F960/T962}$ cells was only 20–40% of that in CHO/IR cells and only about twice that detected in insulin-treated CHO/NEO cells (Fig. 1). Since purified PtdIns 3-kinase will phosphorylate PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2, the relative ability of the anti-Tyr(P) immunoprecipitates from CHO/$IR_{F960/T962}$ and CHO/IR cells to phosphorylate these three substrates was investigated (Table 1). All three activities were reduced in the CHO/$IR_{F960/T962}$ cell immunoprecipitates to approximately the same extent. These results indicate that the F960/T962 mutations do not differentially affect utilization of specific substrates by the activated PtdIns 3-kinase. They also suggest either that less PtdIns 3-kinase is tyrosine phosphorylated in the CHO/$IR_{F960/T962}$ cells or that the phosphorylated protein is less active.

[1] The following abbreviations are used: PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol-phosphate; PtdInsP_2, phosphatidylinositol-bisphosphate; PtdIns(3)P, phosphatidylinositol-3-phosphate; PtdIns(4)P, phosphatidylinositol-4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PtdIns 3-kinase, phosphoinositide 3-(hydroxy)kinase; PtdIns(3,4)P_2, phosphatidylinositol-3,4-bisphosphate; PtdInsP_3, phosphatidylinositol trisphosphate; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor; IGF-I, insulin like growth factor-I; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PS, phosphatidylserine.

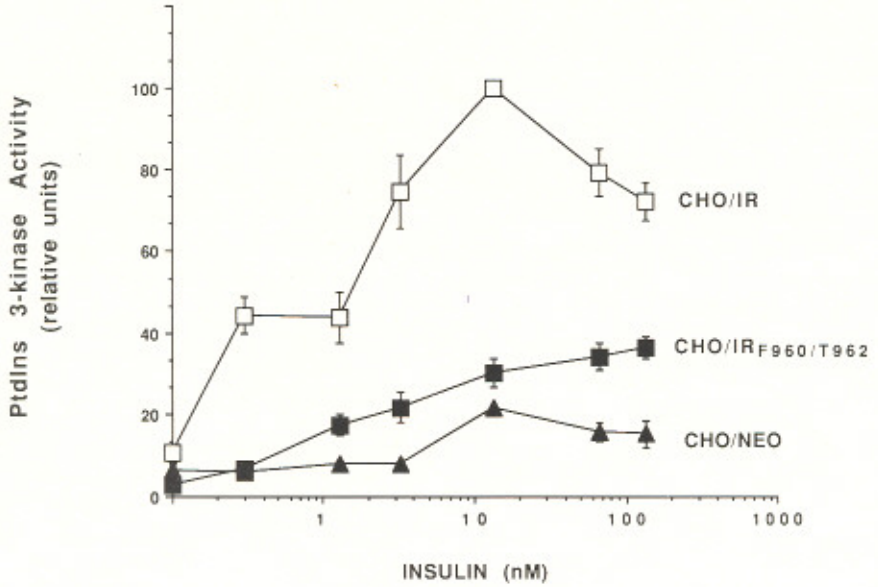

Fig. 1. PtdIns 3-Kinase Activity in Anti-Tyr(P) Immunoprecipitates of Insulin-Stimulated Cells

CHO/IR (□), CHO/$IR_{F960/T962}$ (■), or CHO/NEO (▲) cells were incubated for 5 min with the concentration of insulin indicated. The cell lysates were immunoprecipitated with anti-Tyr(P) antibodies, and PtdIns kinase activity was assayed directly in the immunoprecipitates, as described in *Materials and Methods*. The lipids were separated by TLC, and the radioactive reaction products corresponding to PtdInsP standard were excised and quantitated by scintillation counting. HPLC analysis confirmed that the product was PtdIns(3)P in all three cell types. In each experiment immunoprecipitates were prepared from the three cell types (matched for cell number) in parallel. All data were normalized to the maximal response detected in the CHO/IR cells. The averages and SE of data points from four to six separate experiments are presented. In a typical experiment the 100% value was approximately 10,000 Cherenkov cpm.

Comparison of PtdIns 3-Kinase Activity in Anti-Tyr(P) Immunoprecipitates of CHO Cells Treated with IGF-I and/or Insulin

The IGF-I receptor shows extensive homology in amino acid sequence and structure to the insulin receptor, and shares similar bioactivity (20, 21), including the ability to activate PtdIns 3-kinase (1). It binds both IGF-I and insulin, but with very different affinities (K_d of IGF-I, 1.5×10^{-9} M; K_d of insulin, 10^{-7} M, respectively) (22). The parental CHO cells and the two transfectants used in the present study express approximately 10,000 IGF-I receptors/cell. To test whether there is a postreceptor defect in PtdIns 3-kinase function in CHO/$IR_{F960/T962}$ cells, PtdIns 3-kinase activity was assayed in anti-Tyr(P) immunoprecipitates from IGF-I-stimulated CHO/IR, CHO/$IR_{F960/T962}$, and CHO/NEO cells (Fig. 2). As shown in Fig. 2 IGF-I at concentrations of 10 and 100 nM caused a 4- to 7-fold increase in anti-Tyr(P)-immunoprecipitable PtdIns 3-kinase activity in all three cell lines. In a separate study we found that IGF-I (10 or 100 nM) was more effective than insulin (1 nM) in stimulating anti-Tyr(P)-precipitable PtdIns 3-kinase in both the CHO/$IR_{F960/T962}$ and CHO/NEO cells (data not shown), but less effective than insulin in the CHO/IR cells (1).

These results support our previous conclusion that stimulation of PtdIns 3-kinase activity by insulin is mediated through its high affinity receptor and not through the IGF-I receptor (1). They also indicate that the dual mutation at Tyr960 and Ser962 of the insulin receptor impairs the activation of PtdIns 3-kinase by the insulin receptor, but not by the IGF-I receptor.

Table 1. Relative Utilization of PI, PI-4-P, and PI-4,5-P_2 by Kinase Associated with Anti-Tyr(P) Immunoprecipitates from Insulin-Stimulated CHO/IR and CHO/$IR_{F960/T962}$ Cells

Cell Line	PtdIns(3)P*	PtdIns(3,4)P_2 [% of PtdIns(3)P]	PtdInsP_3 [% of PtdIns(3)P]
CHO/IR	100	32 ± 2	33 ± 4
CHO/$IR_{F960/T962}$	100	43 ± 8	35 ± 10

Experimental conditions were analogous to those described in Fig. 1. Cells were stimulated with 10 nM insulin for 5 min at 37 C and then lysed in 1% Nonidet P-40. Immunoprecipitation with anti-Tyr(P) antibodies was performed as described in *Materials and Methods*. A sonicated mixture of PtdIns, PtdIns(4)P, PtdIns(4,5)P_2, and PS (2:1:1:1; 0.5 mg/ml total) was provided as substrates for the lipid kinase reactions, which were performed directly in the precipitates as described in *Materials and Methods*. PtdIns(3,4)P_2 and PtdInsP_3 produced in the reaction are expressed as a percentage of the ^{32}P incorporation into PtdIns(3)P, generated in the same reaction. The data depicted are the mean ± SE of three separate experiments.

* The total ^{32}P incorporated into PtdIns(3)P was approximately 8000 Cerenkov cpm using immunoprecipitates from CHO/IR cells and approximately 3000 Cerenkov cpm using immunoprecipitates from CHO/$IR_{F960/T962}$ cells.

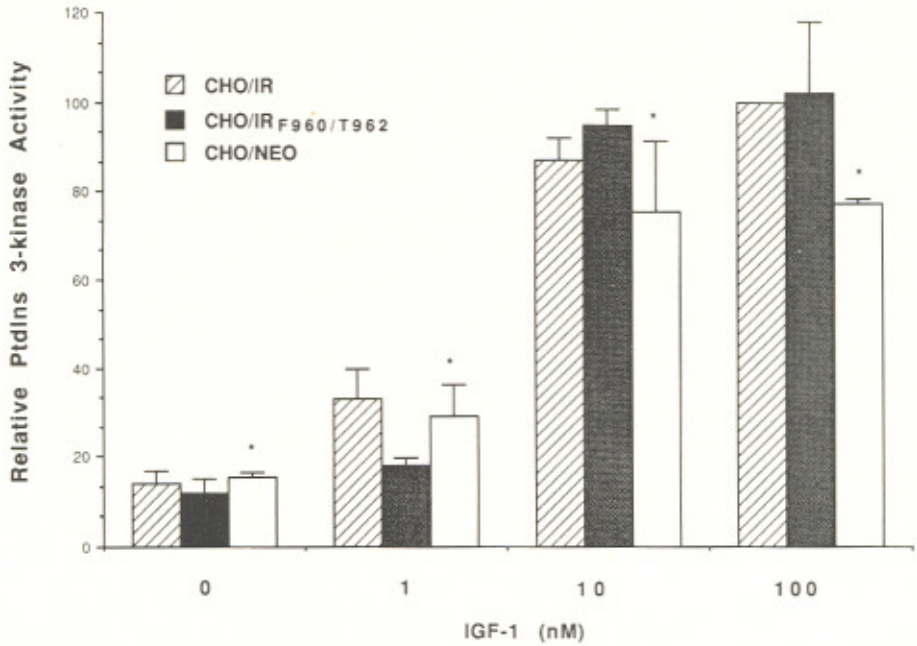

Fig. 2. PtdIns 3-Kinase Activity in Anti-Tyr(P) Immunoprecipitates of Cells Treated with IGF-I

The experiments were performed as described in Fig. 1. The concentration of IGF-I added to intact cells is indicated. Data are presented as a percentage of the maximum value of PtdIns 3-kinase activity observed in anti-Tyr(P) immunoprecipitates of CHO/IR cells stimulated with 100 nM IGF-I. The values for CHO/IR and CHO/$IR_{F960/T962}$ represent data obtained in three different experiments and are expressed as the mean ± SE. The 100% value was approximately 3000 Cherenkov cpm in a typical experiment. ★, n = 2.

Assessment of the 85-kDa Subunit of PtdIns 3-Kinase in Anti-Tyr(P) Immunoprecipitates of Insulin-Stimulated CHO/IR, CHO/$IR_{F960/T962}$, and CHO/NEO Cells by Polyoma Middle T Blotting

Recently, it has been shown that the 85-kDa subunit of PtdIns 3-kinase can be visualized by transferring SDS-denatured protein onto nitrocellulose and blotting with ^{32}P-labeled polyoma middle T-antigen, which is phosphorylated on Tyr315 (6, 17, 18). This is the only protein detected when whole cell lysates from a variety of tissues are probed by this technique (17, 18). We used this technique to estimate the amount of 85-kDa protein in total cell lysates and in anti-Tyr(P) immunoprecipitates of cells expressing either the wild-type (CHO/IR) or the mutant insulin receptor (CHO/$IR_{F960/T962}$). An autoradiograph of a representative blot is shown in Fig. 3. Lane 1 in both panels corresponds to NIH-3T3 cell lysates, which were used as standards due to the previous identification of the 85-kDa protein in these cells by middle T blotting (18). An 86-kDa band appeared with similar intensity in the total cell lysates of all three cell types, indicating that they have similar amounts of this peptide. Insulin addition did not significantly affect the amount of blotted peptide in whole cell lysates. In contrast, in anti-Tyr(P) immunoprecipitates, two bands with apparent mol wt of 86,000 and 84,000 were detected with ^{32}P-labeled middle T only in insulin-treated samples. Furthermore, the intensity of these bands was greater in insulin-stimulated CHO/IR than in insulin-stimulated CHO/$IR_{F960/T962}$ or CHO/NEO cells [the bands in lanes 5 (CHO/$IR_{F960/T962}$) and 7 (CHO/NEO) have approximately 40% and 20%, respectively, of the intensity of the band in lane 3 (CHO/IR)]. This occurred despite the fact that similar amounts of insulin receptor were immunoprecipitated with anti-Tyr(P) antibodies after insulin treatment of CHO/IR and CHO/$IR_{F960/T962}$ cells (data not shown). We have also attempted to detect the 85-kDa protein in antiinsulin receptor immunoprecipitates of CHO/IR and CHO/$IR_{F960/T962}$ cells by this method; however, the amount of 85-kDa protein present in this immunoprecipitates was below the detection level (data not shown). This result is in accordance with sequential immunoprecipitation experiments in which lysates of insulin-stimulated or unstimulated cells were first immunoprecipitated with antiinsulin receptor antibodies, and the supernatants of this precipitates were subsequently immunoprecipitated with anti-Tyr(P) antibodies. In agreement with previous results, the insulin receptor-associated PtdIns 3-kinase activity was only 8.8% of the anti-Tyr(P)-associated activity using CHO/IR cells (1). Similarly, using CHO/$IR_{F960/T962}$ cells, the receptor-associated activity was 10.5% of the anti-Tyr(P)-associated activity. These results are consistent with the inability to detect the 85-kDa protein in the antireceptor immunoprecipitates using the middle T blotting technique. Thus, in both cell types most of the anti-Tyr(P)-associated PtdIns 3-kinase is not complexed with the insulin receptor. A defect in tyrosine phosphorylation of either PtdIns 3-

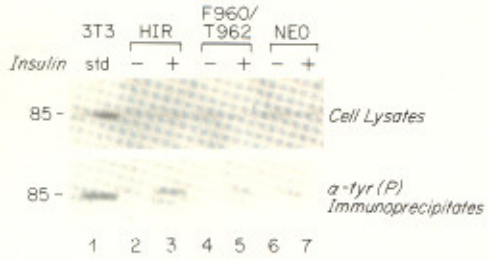

Fig. 3. Detection of the 85-kDa Subunit of PtdIns 3-Kinase in Cell Lysates and Anti-Tyr(P) Immunoprecipitates by Middle T Blotting

Cells were stimulated with insulin (10 nM) where indicated. The probe solution containing ^{32}P-labeled middle T was prepared as described in *Materials and Methods*. Lane 1 corresponds to an aliquot of the cell lysate obtained from NIH/3T3 cells in which pp85 is known to be blotted by tyrosine-phosphorylated middle T and is shown as a positive control. Aliquots containing 100 μg protein were removed from the total cell lysates of CHO/IR, CHO/IR$_{F960/T962}$, and CHO/NEO cells, separated by SDS-PAGE, electroblotted to nitrocellulose, and probed with ^{32}P-labeled middle T (*upper panel*, lanes 2–7). Anti-Tyr(P) immunoprecipitates from the same cell lysates were analyzed by the same procedure (*lower panel*, lanes 2–7). Two bands (apparent mol wt of 86,000 and 84,000) visualized by the ^{32}P-labeled middle T probe appeared only in anti-Tyr(P) immunoprecipitates of insulin-treated cells. The intensities of these bands in lanes 5 and 7 (as quantitated by laser densitometry) were approximately 40% and 20%, respectively, of that detected in lane 3 (*lower panel*).

kinase or an associated protein appears to occur in the CHO/IR$_{F960/T962}$ cells.

Analysis of Polyphosphoinositides in Intact CHO/IR, CHO/IR$_{F960/T962}$, and CHO/NEO Cells after Insulin Stimulation

To investigate further the effect of the mutations at the juxtamembrane region of the insulin receptor on activation of PtdIns 3-kinase by insulin, we studied the labeling of D-3 phosphorylated polyphosphoinositides in intact cells. Previously, we had found that insulin stimulates an increase in ^{32}P incorporation into PtdIns(3,4)P_2 and PtdInsP_3, but not into PtdIns(3)P, in intact CHO/IR cells. The maximum response was reached within 5 min of insulin stimulation (1).

In the present study, cells were prelabeled with [^{32}P] orthophosphate for 3 h at 37 C and then treated with insulin (10 nM) for 5 min. The lipids were extracted and deacylated, and the glycerophosphoinositol polyphosphates were analyzed by anion exchange HPLC, as previously described (5, 23, 24). In CHO/IR cells insulin increased ^{32}P incorporation into PtdIns(3,4)P_2 and PtdInsP_3 by 12- and 6-fold, respectively (Fig. 4). In contrast, insulin did not significantly increase ^{32}P incorporation into these phospholipids in CHO/NEO cells. In CHO/IR$_{F960/T962}$ cells insulin also failed to stimulate ^{32}P incorporation into PtdIns(3,4)P_2; however, its effect on ^{32}P incorporation into PtdInsP_3 was intermediate between that in CHO/IR and CHO/NEO cells. The incorporation of ^{32}P into PtdIns(4)P, PtdIns(3)P, and PtdIns(4,5)P2 was not affected by insulin in any of the three cell lines, in keeping with previous observations (1).

DISCUSSION

The results indicate that mutations in the juxtamembrane region of the insulin receptor impair activation of PtdIns 3-kinase by insulin. Thus, in cells expressing an insulin receptor in which tyrosine-960 and serine-962 are replaced by phenylalanine and threonine, respectively (CHO/IR$_{F960/T962}$), anti-Tyr(P)-immunoprecipitable PtdIns 3-kinase activity was only 20–40% of that in CHO/IR after insulin stimulation. In contrast, stimulation of PtdIns 3-kinase activity in CHO/IR$_{F960/T962}$ cells by IGF-I was normal, indicating that PtdIns 3-kinase can be activated in these cells. Moreover, the amount of the 85-kDa subunit of PtdIns 3-kinase detected by middle T blotting was substantially less in the anti-Tyr(P) immunoprecipitates from insulin-stimulated CHO/IR$_{F960/T962}$ cells, suggesting that mutations in the juxtamembrane region of the insulin receptor reduce the ability of the insulin receptor to phosphorylate PtdIns 3-kinase or an associated protein.

The middle T blotting procedure used to quantitate PtdIns 3-kinase protein has previously been shown to be highly specific for visualizing the 85-kDa subunit of PtdIns 3-kinase (6, 17, 18). Instead of a single band at 85 kDa, as seen in mouse 3T3 fibroblasts (17, 18), we detected two bands with apparent mol wt of 86,000 and 84,000 in CHO cells. Cohen *et al.* (17) previously reported that in Rous sarcoma virus-transformed turkey embryo fibroblasts, two peptides of approximately 85,000 and 86,000 Da associate with middle T. Whether the appearance of two distinct bands in the 85,000-Da region in some tissues is a consequence of differences in species, tissue-specific gene expression, protein modification, proteolysis, or some other factor is not known.

The juxtamembrane region of the insulin receptor may be involved in the recognition of cellular substrates (19). Extensive characterization of the CHO/IR$_{F960/T962}$ cells has revealed defects in insulin-stimulated glycogen synthase activity, amino acid uptake, thymidine incorporation into DNA, and *in vivo* phosphorylation of pp185 (19). Yet this receptor features normal insulin binding, insulin-dependent autophosphorylation, insulin stimulation of phosphotransferase activity toward exogenous substrates *in vitro*, and insulin-dependent internalization (19, 25, 26). The fact that this receptor fails to fully activate the PtdIns 3-kinase *in vivo* raises the possibility that a product of this enzyme [in particular, PtdIns(3,4)P_2] mediates one of the defective responses.

The IR$_{F960/T962}$ protein was originally thought to be mutated only at Tyr960 (19), but subsequent sequence analysis of the gene revealed a second mutation in this

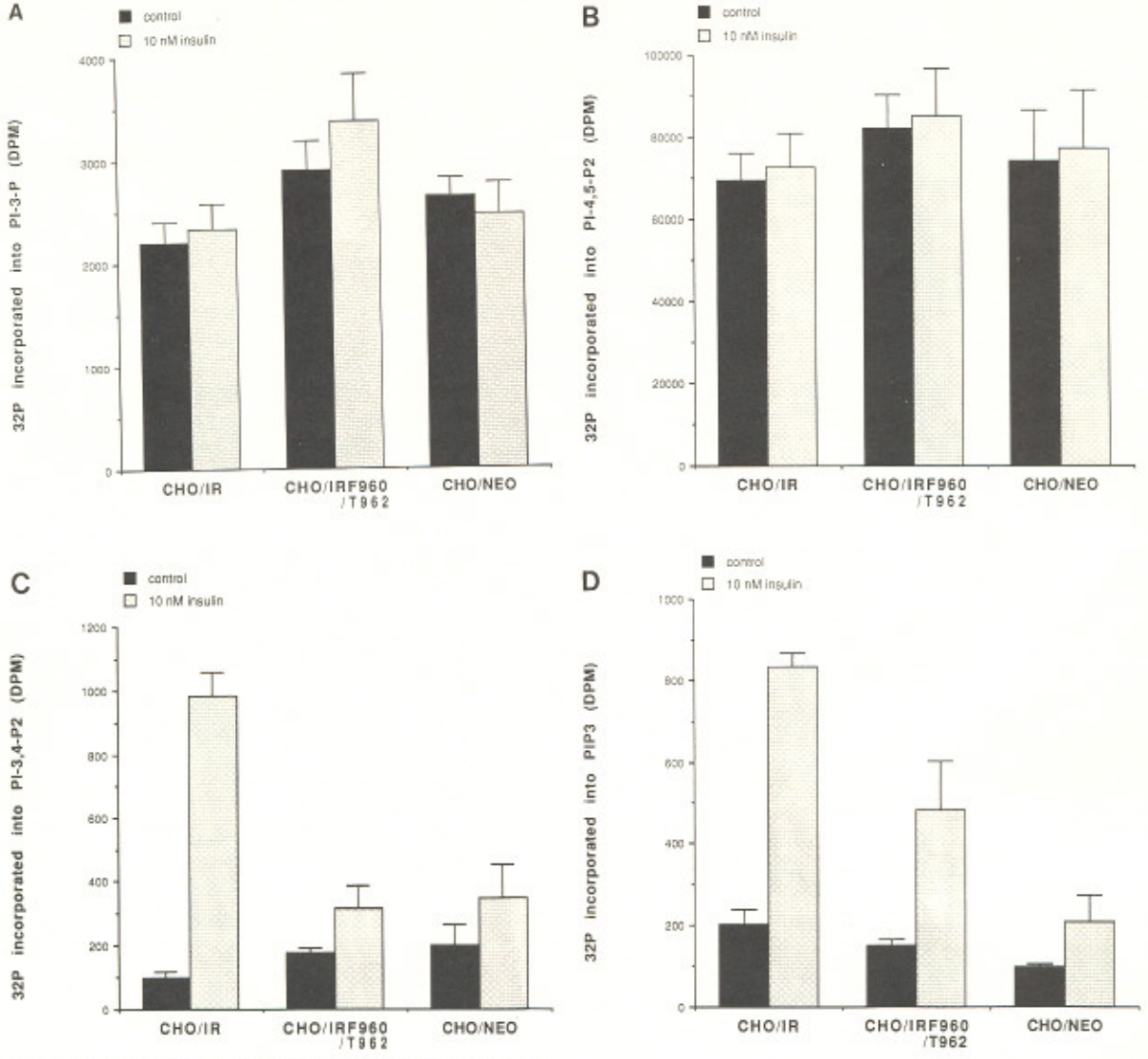

Fig. 4. Polyphosphoinositide Levels in Insulin-Stimulated Cells

Quiescent cells were incubated for 3 h in phosphate-free RPMI medium containing 0.5% albumin and [^{32}P]orthophosphate (100 mCi/ml). Insulin (10 nM) was added where indicated for 5 min. The cells were harvested in 1.2 ml 1 M HCl-methanol-chloroform (1:1:1), the lipid-containing phases deacylated, and the glycerophosphoinositol polyphosphates analyzed by HPLC, as described in *Materials and Methods*. Data are from four experiments and are expressed as the mean ± SE. Each experiment included all three cell types labeled in parallel, with comparable cell numbers with and without exposure to insulin. To average data from the separate experiments, the data were normalized to PI-4-P (typically 1×10^5 dpm): the amount of label incorporated into this lipid was comparable in all three cell types and was unaffected by the 5-min insulin treatment (1). Normalization to total ^{32}P incorporated into polyphosphoinositides produced a similar result and did not significantly affect the average changes observed.

region Ser962 to Thr (White, M., unpublished results). This region of the insulin receptor does not have significant similarity with the consensus sequence for PtdIns 3-kinase association (3). The only region in the insulin receptor that exhibits a weak similarity to the sequence implicated in binding PtdIns 3-kinase surrounds Tyr1322 (3), which is a known phosphorylation site (27). Also, there is no evidence for phosphorylation of this region of the insulin receptor *in vivo* (19). Thus, it seems unlikely that the mutations at Tyr960 and Ser962 directly alter a PtdIns 3-kinase-binding site, as has been implicated for Tyr751 of the PDGF receptor (14, 28). Interestingly, Chen *et al.* (29) recently pointed out that the insulin receptor has a cytosolic region with location and sequence ($NPXY_{960}$) satisfying the rules for coated vesicle-mediated internalization. Tyr960 is in this region, raising the possibility that mutations in this region affect interactions with cellular proteins involved in internalization. Indeed, deletions in this region of the insulin receptor impair insulin-dependent internalization (26, 30). However, as predicted by the rules of Chen *et al.* (29), the F960/T962 double mutation does not impair internalization (26). Thus, the defects in signalling cannot be simply explained by internalization defects. How-

7% SDS-polyacrylamide gel. Aliquots of total cell lysates (100 mg) were run in parallel on the same gel. After separation on SDS-PAGE, the proteins were transferred to nitrocellulose by electroblotting. For this purpose, the nitrocellulose was washed twice in buffer containing 0.32% SDS, 2% Triton X-100, 40 mM triethanolamine (pH 7.4), 80 mM NaCl, and 2 mM EDTA, followed by blocking with 1% gelatin in the same buffer for 30–60 min. Preparation of ^{32}P-labeled middle T and the blotting procedure were described in detail previously (17, 18). The probe was prepared by incubating antimiddle T immunoprecipitate from one 100-mm dish of Sf9 cells, which express both middle T and pp60$^{c\text{-}src}$ (33), with [γ-^{32}P]ATP. The immunoprecipitate containing ^{32}P-labeled middle T was boiled in SDS to dissociate pp60$^{c\text{-}src}$ and reimmunoprecipitated with anti-middle T antibody. The immunoprecipitate was then boiled three times in 300 ml solubilization buffer [0.4% SDS, 50 mM triethanolamine-Cl (pH 7.4), 100 mM NaCl, 2 mM EDTA, and 2 mM 2-mercaptoethanol] to extract all of the labeled middle T, and the latter was analyzed by autoradiography after SDS-gel electrophoresis to confirm the purity of middle T. To final middle T extract was added 0.25 vol 10% Triton X-100 and 0.5 M iodoacetamide to a final concentration of 10 mM. The mixture was then diluted in 10–15 ml solubilization buffer containing 0.25 vol Triton X-100, but lacking 2-mercaptoethanol. After this, the probe-containing solution was incubated for 12 h at 4 C with the nitrocellulose containing the electrotransfers of the anti-Tyr(P) immunoprecipitates. The nitrocellulose was then washed three to five times for 20 min each in the same buffer used for diluting the probe. After air drying, the nitrocellulose was exposed on XAR5 film. The bands were quantitated by laser densitometry.

Detection of Polyphosphoinositides in Intact Cells

Cells that had reached 90% confluence were cultured overnight in serum-free Ham's F-12 medium containing 0.5% albumin. The cells were washed once for 15 min in phosphate-free RPMI medium containing 0.5% albumin and then incubated at 37 C for 3 h in 5 ml of the same medium containing carrier-free [^{32}P]orthophosphate (100 mCi/ml). Insulin (10 nM final concentration) or diluent was then added to the medium, and after 5 min the cells were washed twice with ice-cold buffer A and lysed in 1 M HCl-methanol (1:1, vol/vol), and the lipids were extracted with chloroform. Deacylation and HPLC analysis of the glycerol phosphoinositides were performed as previously described (5, 23).

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