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Insulin Stimulation of Phosphatidylinositol 3-Kinase Activity Maps to Insulin Receptor Regions Required for Endogenous Substrate Phosphorylation*

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We have studied the phosphatidylinositol 3-kinase (PtdIns 3-kinase) in insulin-stimulated Chinese hamster ovary (CHO) cells expressing normal (CHO/IR) and mutant human insulin receptors. Insulin stimulation of CHO/IR cells results in an increase in PtdIns 3-kinase activity associated with anti-phosphotyrosine (αPY) immunoprecipitates, which has been previously shown to correlate with the *in vivo* production of PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 (Ruderman, N., Kapeller, R., White, M. F., and Cantley, L. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 1411-1415). Stimulation was maximal within 1 min and showed a dose response identical to that of insulin receptor autophosphorylation. The PtdIns 3-kinase also associated with the insulin receptor in an insulin-stimulated manner, as approximately 50% of the total αPY-precipitable activity could be specifically immunoprecipitated with anti-insulin receptor antibody. Mutant insulin receptors displayed variable ability to stimulate the PtdIns 3-kinase, but in all cases the presence of PtdIns 3-kinase in αPY immunoprecipitates correlated closely with the tyrosyl phosphorylation of the endogenous substrate pp185. In CHO cells expressing a kinase-deficient mutant (IR_{A1018}), there was no observable insulin stimulation of PtdIns 3-kinase activity in αPY immunoprecipitates and no tyrosyl phosphorylation of pp185. Substitution of Tyr^{1146} in the insulin receptor regulatory region with phenylalanine partially impaired receptor autophosphorylation, pp185 phosphorylation, and insulin-stimulated increases in αPY-precipitable PtdIns 3-kinase activity. In contrast, a deletion mutant lacking 12 amino acids from the juxtamembrane region ($IR_{Δ960}$) displayed normal *in vivo* autophosphorylation but failed to stimulate the PtdIns 3-kinase or phosphorylate pp185. Finally, a mutant receptor from which the C-terminal 43 amino acids had been deleted ($IR_{ΔCT}$) exhibited normal insulin-stimulated autophosphorylation, pp185 phosphorylation, and stimulation of the PtdIns 3-kinase activity in αPY immunoprecipitates. These data suggest that the PtdIns 3-kinase is itself a substrate of the insulin receptor kinase or associates preferentially with a substrate. A comparison of the biological activities of the mutant receptors with their activation of the PtdIns 3-kinase furthermore suggests that the PtdIns 3-kinase may be linked to insulin's ability to regulate DNA synthesis and cell growth.

Insulin binding regulates cellular growth and metabolism through activation of the tyrosine kinase in the β-subunit of the insulin receptor (Kahn and White, 1988). Insulin-stimulated receptor autophosphorylation activates the kinase leading to tyrosyl phosphorylation of endogenous substrates which are thought to play a role in signal transmission (Kasuga *et al.*, 1982; Rosen *et al.*, 1983; White *et al.*, 1985). Recent work in this laboratory and others (Wilden *et al.*, 1990; Maegawa *et al.*, 1988) suggests that the insulin signal is mediated through multiple signal transduction pathways. One of these pathways may involve a 185-kDa phosphotyrosine-containing protein, pp185 (White *et al.*, 1985). pp185 is found in insulin-stimulated cells by immunoprecipitation or immunoblotting with the anti-phosphotyrosine antibody, and migrates in reducing SDS-PAGE[1] gels as a diffuse band which may contain several polypeptide species.[2] A component of pp185 has recently been purified and cloned (Rothenberg *et al.*, 1991; Sun *et al.*, 1991). The cloned protein, named IRS-1 (insulin receptor substrate 1), contains a number of potential serine/threonine and over 10 potential tyrosine phosphorylation sites, six of which are in YMXM motifs.

A second insulin-signaling pathway may involve the activation of the phosphatidylinositol (PtdIns) 3-kinase (Whitman *et al.*, 1985, 1988; Auger *et al.*, 1989). The PtdIns 3-kinase is found in anti-phosphotyrosine antibody immunoprecipitates from insulin-stimulated cells, suggesting that it undergoes tyrosyl phosphorylation (Ruderman *et al.*, 1990; Endemann *et al.*, 1990). Like insulin-stimulated phosphorylation of pp185, insulin stimulation of the PtdIns 3-kinase occurs rapidly and at physiological concentrations of insulin (Ruderman *et al.*, 1990; Endemann *et al.*, 1990) and can be

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[1] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IRS-1, insulin receptor substrate 1; PtdIns, phosphatidylinositol; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; CSF, colony-stimulating factor; αPY, anti-phosphotyrosine; IR, insulin receptor; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

[2] M. Miralpeix and M. F. White, unpublished results.

detected *in vivo* in ^{32}P-labeled cells (Ruderman *et al.*, 1990). Substrates like pp185 and the PtdIns 3-kinase may therefore be involved in the early steps of insulin signal transmission.

The PtdIns 3-kinase is activated by a number of tyrosine kinases including the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), colony-stimulating factor (CSF), and insulin receptors, as well as the pp60$^{c\text{-}src}$/polyoma middle T complex (Whitman *et al.*, 1988; Coughlin *et al.*, 1989; Varticovski *et al.*, 1989; Bjorge *et al.*, 1989; Pignataro and Ascoli, 1990; Ruderman *et al.*, 1990; Endemann *et al.*, 1990). The PtdIns 3-kinase appears to be a heterodimer composed of an 85-kDa subunit which is thought to play a regulatory role and a 110-kDa presumed catalytic subunit (Carpenter *et al.*, 1990; Shibasaki *et al.*, 1991). The activated PtdIns 3-kinase phosphorylates phosphatidylinositol and its derivatives at the D3 position of the inositol ring, producing *in vivo* PtdIns-3P, PtdIns-3,4-P_2, and PtdIns-3,4,5-P_3 (Whitman *et al.*, 1988; Auger *et al.*, 1989). Although the PtdIns 3-kinase does not appear to show substrate specificity for PtdIns, PtdIns(4)P, or PtdIns(4,5)P_2 *in vitro*, recent data suggest that the primary *in vivo* substrate is PtdIns(4,5)P_2, with the other products arising from successive dephosphorylations (Stevens *et al.*, 1991); these results, however, have been controversial (Majerus *et al.*, 1990; Carpenter and Cantley, 1990). The lipid products can be dephosphorylated by a specific lipid phosphatase, but they are not substrates for PtdIns-specific phospholipase C_γ and their role in signaling is not known (Lips *et al.*, 1989; Lips and Majerus, 1989).

The presence of PtdIns 3-kinase activity in anti-phosphotyrosine and anti-insulin receptor immunoprecipitates from insulin-stimulated cells suggests that the PtdIns 3-kinase is a direct substrate of the insulin receptor kinase or associates with the receptor or another protein that undergoes tyrosine phosphorylation during insulin stimulation. In this regard, we have recently shown that the PtdIns 3-kinase associates strongly with IRS-1, presumably through phosphorylated YM*X*M residues (Sun *et al.*, 1991); the role of this association in the activation of the PtdIns 3-kinase is not yet clear. Furthermore, the relationship between the pools of PtdIns 3-kinase detected with anti-phosphotyrosine, anti-receptor, and anti-IRS-1 antibodies is not fully understood. In the present study, we have restricted our attention to the effect of insulin receptor mutations on the association of the PtdIns 3-kinase with αPY and antireceptor immunoprecipitates, which has been shown to correlate with *in vivo* activation of the enzyme (Ruderman *et al.*, 1990). Our data suggest that stimulation of the PtdIns 3-kinase by the insulin receptor requires an intact ATP binding site, and residues in the juxtamembrane and kinase-regulatory regions, but does not require residues in the C terminus of the receptor. Moreover, immunoprecipitation of the PtdIns 3-kinase by αPY correlates with αPY immunoprecipitation of pp185, rather than with autophosphorylation of the insulin receptor itself. Therefore, the PtdIns 3-kinase may be a substrate of the insulin receptor kinase with a similar specificity to that of pp185 or may be associated with pp185. Its activation may play an important role in the insulin stimulation of cell growth.

MATERIALS AND METHODS

Transfection of CHO Cells—The normal human insulin receptor (IR) expression plasmid pCVSVHIRc and the expression plasmid encoding mutant human insulin receptors IR_{A1018}, $IR_{\Delta 960}$, IR_{F1146}, and $IR_{\Delta CT}$ have been described previously (Chou *et al.*, 1987; Backer *et al.*, 1990; Wilden *et al.*, 1990; McClain *et al.*, 1987). CHO cells were grown in 10-cm dishes in F-12 medium containing 10% fetal bovine serum (GIBCO) and transfected as described previously (White *et al.*, 1988). CHO cell lines that expressed high levels of surface insulin receptors were selected by fluorescence-activated cell sorting (Maron *et al.*, 1984), and clonal cell lines were obtained by plating at limiting dilution. The clonal lines of CHO/IR cells and mutant CHO/IR_{F960}, CHO/$IR_{\Delta 960}$, CHO/IR_{A1018} and CHO/IR_{F1146} cells expressed approximately 10^6 receptors/cell; clonal lines of $IR_{\Delta CT}$ cells expressed approximately 50% fewer receptors. Scatchard analysis indicated that insulin binding affinity was normal in all lines (Backer *et al.*, 1990 and data not shown).

Antibodies—Polyclonal rabbit antibodies to phosphotyrosine and IRS-1 were prepared as described previously (White and Backer, 1991; Sun *et al.*, 1991). Polyclonal human anti-insulin receptor antibodies from patient sera were prepared as described previously (Kahn *et al.*, 1981). Mouse monoclonal antireceptor antibodies were provided by Dr. K. Siddle, University of Cambridge, United Kingdom.

[^{32}P]Phosphate Labeling of CHO Cells Expressing Wild-type Insulin Receptors—Confluent monolayers of transfected CHO cells in 10- or 15-cm dishes (Nunc) at 37 °C were labeled for 2 h with 0.5 mCi/ml with [^{32}P]phosphate (Du Pont-New England Nuclear) as described previously (Backer *et al.*, 1990). The cells were incubated for additional periods of time in the presence of 100 nM insulin, rapidly frozen with liquid nitrogen, and solubilized in 100 mM Tris, pH 8.0, containing 2 mM sodium vanadate, 0.34 mg/ml phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 1 μg/ml leupeptin, and 1% Triton X-100. Tyr(P)-containing proteins were immunoprecipitated with anti-phosphotyrosine antibody (αPY), and precipitated proteins were reduced with dithiothreitol and analyzed by SDS-PAGE (White and Backer, 1991). Immunoprecipitated proteins were identified by autoradiography, and the radioactivity in the insulin receptor subunits was quantified by liquid scintillation counting.

In Vitro Insulin Receptor Autophosphorylation—Insulin receptors were purified on wheat germ agglutinin-agarose (Vector). Aliquots of receptor, normalized to binding, were incubated in 50 μl of 50 mM HEPES, pH 7.5, containing 0.1% Triton X-100, 5 mM $MnCl_2$, and varying concentrations of insulin. The reaction was initiated by the addition of 30 μCi of [^{32}P]ATP (Du Pont-New England Nuclear) in the presence of 25 μM unlabeled ATP. The autophosphorylation reactions were terminated after 10 min at 20 °C by boiling in Laemmli sample buffer containing 100 mM dithiothreitol (Laemmli, 1970); alternatively, samples were immunoprecipitated with αPY or anti-insulin receptor antibodies (αIR) and eluted by boiling as above. The samples were separated by reducing SDS-PAGE (6% resolving gels), visualized by autoradiography, and the radioactivity in β-subunit bands was determined by Cerenkov counting.

Phosphatidylinositol 3-Kinase Activity—In vitro phosphorylation of phosphatidylinositol was measured as described previously (Ruderman *et al.*, 1990). Subconfluent CHO cells grown in 100-mm dishes were made quiescent by an overnight incubation in F-12 medium containing 0.5% bovine serum albumin. The cells were then incubated in the absence or presence of insulin (100 nM) for 10 min and washed once with ice cold phosphate-buffered saline and twice with 20 mM Tris, pH 7.5, containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μM Na_3VO_4 (Buffer A). The cells were solubilized in 1 ml of Buffer A containing 1% Nonidet P-40 (Sigma), 10% glycerol, and 0.35 mg/ml phenylmethylsulfonyl fluoride and insoluble material was removed by centrifugation at 13,000 × *g* for 10 min. Tyrosyl phosphoproteins were immunoprecipitated from the supernatant with αPY and protein A-Sepharose (Pharmacia LKB Biotechnology Inc.). Alternatively, anti-insulin receptor or control antibodies were used as described in the text. The immunoprecipitates were washed successively in phosphate-buffered saline containing 1% Nonidet P-40 and 100 μM Na_3VO_4 (three times), 100 mM Tris, pH 7.5, containing 500 mM $LiCl_2$ and 100 μM Na_3VO_4 (three times) and 10 mM Tris, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4 (two times). The pellets were resuspended in 50 μl of 10 mM Tris, pH 7.5, containing 100 mM NaCl and 1 mM EDTA. To each pellet was added 10 μl of 100 mM $MnCl_2$ and 10 μl of phosphatidylinositol (2 μg/μl) sonicated in 10 mM Tris, pH 7.5, 1 mM EGTA. The reaction was started by the addition of 10 μl of 440 μM ATP containing 30 μCi of [^{32}P]ATP. After 10 min at 22 °C, the reaction was stopped by the addition of 20 μl of 8 N HCl and 160 μl of $CHCl_3$:methanol (1:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate (Merck) which had been coated with 1% potassium oxalate. TLC plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2), dried, and visualized by autoradiography. The radioactivity in spots which co-migrated with PtdIns-4P standard (Sigma) was measured by Cerenkov counting as described previously (Ruderman *et al.*, 1990).

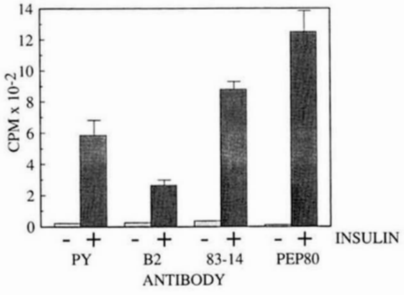

FIG. 1. **PtdIns 3-kinase activity in immunoprecipitates from CHO cells expressing human insulin receptors.** CHO/IR cells were incubated in the absence or presence of 100 nM insulin for 10 min, chilled, solubilized, and immunoprecipitated with a polyclonal αPY antibody, polyclonal (*B2*) and monoclonal (*83-14*) αIR antibodies, or a polyclonal αIRS-1 (*Pep80*) antibody. PtdIns 3-kinase activity in the protein A-Sepharose pellets were assayed as described under "Materials and Methods." Data show the mean ± S.D. of triplicates. Data was not normalized for the relative efficiency of the different antibodies.

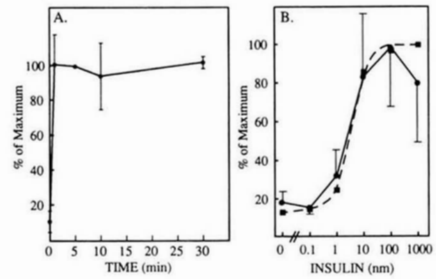

FIG. 2. **Insulin stimulation of PtdIns 3-kinase activity in CHO cells expressing human insulin receptors.** *A*, CHO/IR cells were stimulated with 100 nM insulin for varying times. The cells were chilled, solubilized, and tyrosyl phosphoproteins were immunoprecipitated with αPY and protein A-Sepharose. PtdIns 3-kinase activity in the pellets were assayed as described under "Materials and Methods." Data show the mean ± S.D. of triplicates. *B*, CHO/IR cells were stimulated with varying concentrations of insulin for 10 min, followed by solubilization, immunoprecipitation with αPY, and assay of PtdIns 3-kinase activity as above; data show the mean ± S.D. of triplicates (*solid line*). Partially purified insulin receptors from CHO/IR cells were allowed to autophosphorylate for 5 min at 22 °C in the presence of [^{32}P]ATP and varying concentrations of insulin as described. The phosphorylated receptors were separated by SDS-PAGE and subjected to autoradiography, and incorporation of ^{32}P into β-subunit bands was determined by Cerenkov counting (*dashed line*).

RESULTS

Immunoprcipitation of PtdIns 3-Kinase Activity with Anti-phosphotyrosine, Anti-insulin Receptor, and Anti-IRS-1 Antibodies—CHO cells expressing the human insulin receptor (CHO/IR) were incubated in the absence or presence of insulin and immunoprecipitated with polyclonal antibodies against phosphotyrosine (*αPY*), the human insulin receptor (*B2*) or the insulin receptor substrate IRS-1 (*αpep80*), and a monoclonal antibody against the human insulin receptor (*83-14*). The washed immunoprecipitates were assayed *in vitro* for the presence of PtdIns 3-kinase activity (Fig. 1). Little PtdIns 3-kinase activity was detected in immunoprecipitates from unstimulated cells. αPY and the two αIR immunoprecipitates from insulin-stimulated cells showed a 10–20-fold increase in associated PtdIns 3-kinase activity and an even greater increase was seen in αpep80 immunoprecipitates. Although the immunoprecipitation of PtdIns 3-kinase activity by each of the three antibodies was stimulated by insulin, the degree of overlap between these three pools of PtdIns 3-kinase activity is not yet clear. Furthermore, although the insulin-stimulated association of the PtdIns 3-kinase with αPY and αIR immunoprecipitates correlates with the activation of the enzyme in intact cells (Ruderman *et al.*, 1990), the physiological consequences of the association of the PtdIns 3-kinase with IRS-1 are not yet known. Recent results in fact suggest that the insulin-stimulated pp185 band detected with αPY antibodies contains several Tyr(P) containing proteins, one of which is IRS-1.[3] Thus, the remainder of this study focuses on the insulin-stimulated association of the PtdIns 3-kinase with αIR and αPY immunoprecipitates, and a subsequent report will specifically address the role of IRS-1 in the regulation of the PtdIns 3-kinase.

Time Course and Dose Response of Insulin-stimulated PtdIns 3-Kinase Activity in CHO/IR Cells—PtdIns 3-kinase activity was detected in αPY immunoprecipitates from insulin-stimulated CHO/IR cells; insulin rapidly stimulated the PtdIns 3-kinase by 10–20-fold (Fig. 2*A*). The activity was maximal in under 1 min and remained elevated for up to 30 min. Further characterization of the PtdIns 3-kinase activity was performed after a 10-min insulin stimulation, during the plateau of activation. Immunoprecipitation of the PtdIns 3-kinase with αPY was specific, as it could be blocked with 10 mM *p*-nitrophenylphosphate (Fig. 3*A*). Stimulation of the PtdIns 3-kinase in αPY immunoprecipitates was observed at physiological levels of insulin, with half-maximal activity at 3 nM insulin (Fig. 2*B*, *solid line*). This value is similar to the k_D for insulin binding in CHO/IR cells (Shiymko *et al.*, 1989). Furthermore, the dose response of insulin-stimulated PtdIns 3-kinase activity was virtually identical to that of autophosphorylation of partially purified insulin receptors from the CHO/IR cells (Fig. 2*B*, *dotted lines*). Thus, insulin stimulation of the PtdIns 3-kinase appears to be closely coupled to stimulation of the insulin receptor kinase.

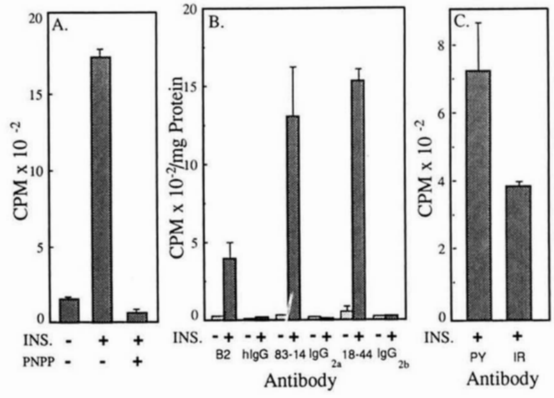

FIG. 3. **Detection of insulin-stimulated PtdIns 3-kinase activity with αPY and anti-insulin receptor antibodies.** CHO/IR cells were incubated in the absence or presence of with 100 nM insulin for 10 min, solubilized, and immunoprecipitated under varying conditions. PtdIns 3-kinase activity in the immunoprecipitates was assayed as above. *A*, immunoprecipitations employed αPY in the absence or presence of 10 mM *p*-nitrophenylphosphate (*PNPP*). *B*, immunoprecipitations employed polyclonal human αIR (*B2*), control human IgG, αIR mouse monoclonal IgG_{2a} (*83-14*), control mouse IgG_{2a}, αIR mouse monoclonal IgG_{2b} (18–44), or control mouse IgG_{2b}. Data show the mean ± S.D. of triplicates. *C*, immunoprecipitations employed αPY or mouse monoclonal anti-insulin receptor (*αIR*) antibody (18-44). In parallel, partially purified insulin receptors from CHO/IR cells were radiolabeled by *in vitro* insulin-stimulated autophosphorylation in the presence of [^{32}P]ATP and mixed with unlabeled solubilized CHO/IR cells, and insulin receptors were immunoprecipitated with αPY or αIR and analyzed by SDS-PAGE. PtdIns 3-kinase activity detected with each antibody was normalized to the efficiency with which each antibody immunoprecipitated a common substrate, the ^{32}P-labeled insulin receptor.

[3] M. Miralpiex and M. F. White, manuscript in preparation.

Association of the Stimulated PtdIns 3-Kinase with the Insulin Receptor—Although the stimulated PtdIns 3-kinase is tightly associated with other tyrosyl kinase receptors such as the PDGF and CSF receptors (Coughlin *et al.*, 1989; Varticovski *et al.*, 1989), variable results concerning the association of the PtdIns 3-kinase with the insulin receptor have been reported (Ruderman *et al.*, 1990; Endemann *et al.*, 1990). We measured insulin receptor-associated PtdIns 3-kinase activity using a polyclonal human and two different monoclonal mouse anti-insulin receptor (αIR) antibodies (Fig. 3*B*). Each antibody detected a 10–20-fold stimulation of receptor-associated PtdIns 3-kinase activity. This activity was significantly greater than that observed using control antibodies which were matched for species and IgG class, indicating that the immunoprecipitation of the PtdIns 3-kinase by αIR was specific.[4]

To compare the receptor-associated activity with the total PtdIns 3-kinase activity measured in αPY immunoprecipitates, we normalized the efficiencies of the αPY and αIR antibodies by measuring their ability to immunoprecipitate a common substrate, the *in vitro* phosphorylated human insulin receptor, under conditions identical to those used for the immunoprecipitation of the PtdIns 3-kinase. When corrected for the efficiency of immunoprecipitation, the amount of receptor-associated PtdIns 3-kinase activity was 50% of the total αPY-precipitable activity (Fig. 3*C*). The enhanced recovery of the PtdIns 3-kinase from insulin-stimulated cells by the αPY relative to αIR is presumably due to direct tyrosine phosphorylation of the PtdIns 3-kinase or its association with another protein that undergoes tyrosine phosphorylation during insulin stimulation, such as IRS-1.

Receptor Autophosphorylation and Phosphorylation of pp185 in Cells Expressing Wild-type and Mutant Insulin Receptors—We examined receptor autophosphorylation, endogenous substrate phosphorylation, and activation of the PtdIns 3-kinase in CHO cells expressing similar numbers of the normal insulin receptors as well as receptors containing mutations in four regions of the cytoplasmic β-subunit. $IR_{\Delta 960}$ contains a 12-amino acid deletion (A954-D965) in the juxtamembrane region (Backer *et al.*, 1990). The ATP binding site mutant IR_{A1018} contains alanine in place of Lys^{1018} (Chou *et al.*, 1987). The regulatory region mutant IR_{F1146} contains phenylalanine in place of Tyr^{1146} (Wilden *et al.*, 1990). Finally, $IR_{\Delta CT}$ is truncated at its C terminus by 43 amino acids, which removes both C-terminal phosphorylation sites (Maegawa *et al.*, 1988; Myers *et al.* 1991). With the exception of the inactive IR_{A1018}, each mutant receptor undergoes insulin-stimulated autophosphorylation in the intact cell (Fig. 4). In particular, the tryptic peptide maps of the autophosphorylated IR and $IR_{\Delta 960}$ are identical (data not shown), whereas the $IR_{\Delta CT}$ undergoes trisphosphorylation of the regulatory region normally but lacks C-terminal phosphorylation (Myers *et al.*, 1991). The IR_{F1146} undergoes altered phosphorylation in the regulatory region, as it lacks one of the three phosphorylation sites, but normal phosphorylation in the C terminus (data not shown). The overall level of insulin-stimulated receptor autophosphorylation is reduced by 50% in the IR_{F1146} cells, whereas it is normal in the $IR_{\Delta 960}$ cells and proportionally reduced in the $IR_{\Delta CT\ cells}$ due to the removal of two phosphorylation sites (Backer *et al.*, 1991; Myers *et al.*, 1991).

Insulin-stimulated tyrosyl phosphorylation of pp185, detected in αPY immunoprecipitates, varied greatly between cells expressing different receptor mutants. Phosphorylated pp185 cannot be detected in αPY immunoprecipitates from [^{32}P]phosphate-labeled CHO/neo cells, whereas it is strongly detected in immunoprecipitates from CHO/IR and CHO/$IR_{\Delta CT}$ cells (Fig. 4, *A* and *C*). In contrast, a slight amount of pp185 phosphorylation was detected in the IR_{F1146} cells, whereas none was detected in the CHO/$IR_{\Delta 960}$ cells (Fig. 4, *A* and *B*). The tyrosyl phosphorylation of an uncharacterized 85–90-kDa protein is also apparent in CHO/IR cells but reduced in CHO/$IR_{\Delta 960}$ and CHO/IR_{F1146} cells (Fig. 4, *A* and *B*); phosphorylation of the 85–90-kDa band in the CHO/$IR_{\Delta CT}$ cells is undetectable due to overlap with the truncated β-subunit band. Thus, insulin-stimulated tyrosyl phosphorylation of multiple proteins is reduced in the CHO/$IR_{\Delta 960}$ and CHO/IR_{F1146} cells, but apparently normal in the CHO/$IR_{\Delta CT}$ cells.

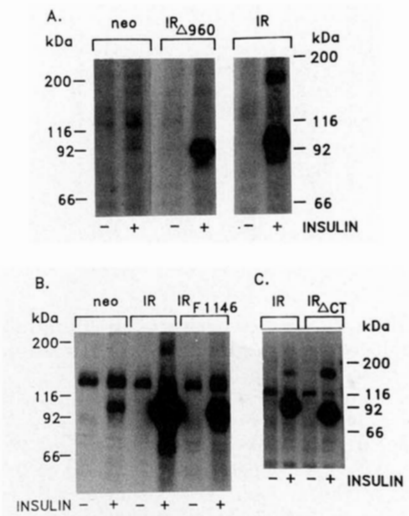

FIG. 4. ***In vivo* phosphorylation of the insulin receptor and its endogenous substrates.** CHO/neo, CHO/$IR_{\Delta 960}$, and CHO/IR cells (*A*); CHO/neo, CHO/IR, CHO/IR_{F1146} cells (*B*); or CHO/IR and CHO/$IR_{\Delta CT}$ cells (*C*) were labeled with [^{32}P]orthophosphate for 2 h and incubated in the absence or presence of 100 nM insulin for 2 min. Tyrosyl-phosphorylated receptors were immunoprecipitated with αPY, separated by SDS-PAGE, and visualized by autoradiography.

Insulin Receptor Mutants Display Variable Stimulation of the PtdIns 3-Kinase—Activation of the PtdIns 3-kinase by these mutant receptors, as detected by the presence of PtdIns 3-kinase activity in αPY immunoprecipitates, was compared with the activity seen in CHO/IR cells and control CHO/neo cells (Fig. 5). Cells expressing the kinase-deficient IR_{A1018}, in which autophosphorylation of the mutant receptor and pp185 is undetectable (data not shown), showed no stimulation of the PtdIns 3-kinase above control cells (Fig. 5*A*). The $IR_{\Delta 960}$ receptor, which undergoes autophosphorylation normally *in vivo* (Fig. 4), also showed no stimulation of the PtdIns 3-kinase above that seen in control CHO/neo cells (Fig. 5*B*). This is consistent with the previously reported finding that the more conservative mutant IR_{F960}, in which Tyr^{960} and Ser^{962} are replaced by Phe and Thr, respectively, also has a reduced capacity to stimulate the PtdIns 3-kinase (Kapeler *et. al.*, 1991). In CHO/IR_{F1146} cells, insulin stimulation of the PtdIns 3-kinase revealed a more complex alteration. We observed a 100-fold decrease in sensitivity and a 50% decrease in maximal activity at high insulin concentrations (Fig. 5*C*). Finally, cells expressing the C-terminal mutant $IR_{\Delta CT}$ showed no defect in the activation of the PtdIns 3-kinase (Fig. 5*D*).

Interestingly, none of the mutants differed significantly from the wild type IR with regard to the percentage of PtdIns 3-kinase activity found in αIR immunoprecipitates relative to αPY immunoprecipitates (Fig. 6). In each case, the amount

[4] The differences between the anti-insulin receptor antibodies is due to different efficiencies of immunoprecipitation which were not normalized in this experiment.

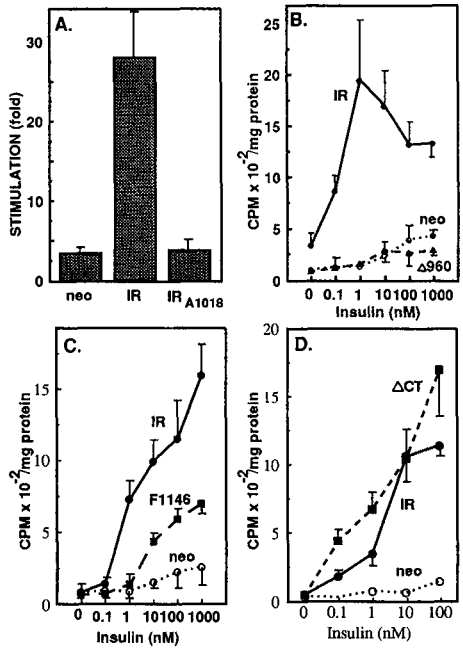

FIG. 5. **Insulin-stimulated PtdIns 3-kinase activity in CHO cells expressing normal and mutant human insulin receptors.** CHO cells expressing normal or mutant insulin receptors were stimulated with 100 nM insulin (*A*) or with varying insulin concentrations (*B–D*) at 37 °C for 10 min. The cells were solubilized and immunoprecipitated with αPY and protein A-Sepharose, and the PtdIns 3-kinase in the protein A pellets was assayed as described above. In each experiment, CHO/IR and control CHO/neo cells were compared with the following mutants: *A*, CHO/IR_{A1018} cells. *B*, CHO/$IR_{\Delta 960}$ cells. *C*, CHO/IR_{F1146} cells. *D*, CHO/$IR_{\Delta CT}$ cells. Data show the mean ± S.D. of triplicates and are representative of two to three separate experiments.

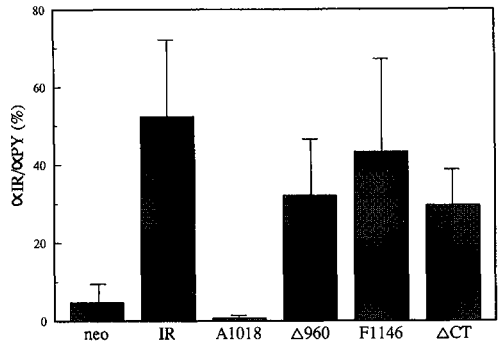

FIG. 6. **Association of the insulin-stimulated PtdIns 3-kinase with insulin receptor mutants.** CHO cells expressing normal or mutant insulin receptors were stimulated with 100 nM insulin. The cells were extracted and the PtdIns 3-kinase in αPY and monoclonal αIR (83-14 or 18-44) immunoprecipitates was determined as described above. Insulin-stimulated PtdIns 3-kinase activity present in αIR immunoprecipitates was plotted as a percentage of that in αPY immunoprecipitates.

of PtdIns 3-kinase associated with the insulin receptor was approximately 50% of that detected in αPY immunoprecipitates.[5] Therefore, the inability of some of the mutants to stimulate the PtdIns 3-kinase did not result from an inability to form stable insulin receptor-PtdIns 3-kinase complexes and may instead reflect a decreased ability of the insulin receptor to phosphorylate the PtdIns 3-kinase or other endogenous substrates on tyrosine residues.

[5] The extremely low αIR/αPY ratios in the CHO/neo and CHO/IR_{A1018} cells are due to the fact that the monoclonal αIR antibody does not recognize the endogenous rodent insulin receptor and do not suggest that the endogenous CHO insulin receptor is unable to associate with the PtdIns 3-kinase.

DISCUSSION

The insulin receptor is one member of a growing class of cellular tyrosyl kinases which activate the PtdIns 3-kinase. Receptors for insulin, PDGF, EGF, CSF-1, as well as the polyoma middle T/$pp60^{c\text{-}src}$ complex modulate the activity of the PtdIns 3-kinase extracted from stimulated cells, and increased levels of PtdIns 3-phospho-derivatives are detected in PDGF-stimulated fibroblasts and activated platelets (Whitman *et al.*, 1985, 1988; Auger *et al.*, 1989; Bjorge *et al.*, 1989; Coughlin *et al.*, 1989; Varticovski *et al.*, 1989; Majerus *et al.*, 1990; Ruderman *et al.*, 1990; Pignataro and Ascoli, 1990). Activation or intracellular translocation of the enzyme apparently involve noncovalent associations between the PtdIns 3-kinase, tyrosyl kinases, and related proteins, and the enzyme can be immunoprecipitated by antibodies directed against the PDGF, CSF-1, and EGF receptors and polyoma middle T (Whitman *et al.*, 1988; Bjorge *et al.*, 1989; Coughlin *et al.*, 1989; Varticovski *et al.*, 1989). These interactions are presumably mediated by the binding of SH-2 domains in the 85-kDa subunit of the PtdIns 3-kinase to phosphotyrosyl residues on associated proteins, which serve as intracellular ligands (Escobedo *et al.*, 1991b; Skolnik *et al.*, 1991; Otsu *et al.*, 1991; Koch *et al.*, 1991).

The activation of the PtdIns 3-kinase may involve tyrosyl phosphorylation of its 85-kDa subunit, since the activity can be detected in anti-phosphotyrosine (αPY) immunoprecipitates (Whitman *et al.*, 1988; Coughlin *et al.*, 1989; Bjorge *et al.*, 1989; Varticovski *et al.*, 1989), and the tyrosyl phosphorylation the 85-kDa subunit correlates with a PDGF-stimulated increase in measurable PtdIns 3-kinase activity (Courtneidge and Heber, 1987; Kaplan *et al.*, 1987; Cohen *et al.*, 1990a). Characterization of pp85 from a variety of cell types demonstrates a family of polypeptides of variable isoelectric points, presumably due to variable phosphorylation on tyrosine and serine residues (Kaplan *et al.*, 1987; Cohen *et al.*, 1990a). Tyrosyl phosphorylation of pp85 was also recently shown to coincide with its translocation from the cytosol to the plasma membrane; the functional consequences of this translocation are not yet known (Cohen *et al.*, 1990b).

Although it is not yet certain whether the tyrosyl or serine phosphorylation of the PtdIns 3-kinase increases its activity or alters its affinity for tyrosyl kinases, clear evidence has emerged showing that tyrosyl phosphorylation of PtdIns 3-kinase binding proteins is crucial for their interactions with the PtdIns 3-kinase. The polyoma middle T antigen and the kinase insert regions of the PDGF and CSF-1 receptors contain tyrosine phosphorylation sites which have been implicated in the binding of the PtdIns 3-kinase (Kazlauskas and Cooper, 1989, 1990; Talmadge *et al.*, 1989; Shurtleff *et al.*, 1990; Reedijk *et al.*, 1990). These sites are present in homologous domains composed of the sequence YM*X*M for polyoma middle T and both YM*X*M and YV*X*M for the PDGF-α, PDGF-β, and CSF-1 receptors (Soeda *et al.*, 1980; Yarden *et al.*, 1986; Coussens *et al.*, 1986). Mutants of the PDGF receptor and polyoma middle T which lack these phosphorylation sites do not bind or activate the PtdIns 3-kinase (Kazlauskas and Cooper, 1989, 1990; Talmadge *et al.*, 1989), and phosphopeptides derived from the PDGF receptor insert domain block PtdIns 3-kinase binding to the receptor (Escobedo *et al.*, 1991a). The EGF receptor also contains a YM*X*M motif, although it may not be phosphorylated and the role of this region of the receptor in binding the PtdIns 3-kinase has not

been established (Bjorge *et al.*, 1989; Ullrich *et al.*, 1984). The proto-oncogene $pp60^{c\text{-}src}$, which does not itself contain the YMXM motif, activates the PtdIns 3-kinase in association with polyoma middle T (Whitman *et al.*, 1985; Kaplan *et al.*, 1986). However, the viral homologue $pp60^{v\text{-}src}$ can apparently activate the PtdIns 3-kinase without the presence of a known intermediary protein (Sugimoto *et al.*, 1984). Recent studies in fact suggest that v-src/PtdIns 3-kinase binding may involve an interaction between the v-src SH-2 domain and phosphorylation sites in the PtdIns 3-kinase, an apparent inversion of the usual pattern (Fukui and Hanafusa, 1991).

Like the PDGF, CSF-1 and EGF receptors, the insulin receptor appears to both activate and bind to the PtdIns 3-kinase upon stimulation with ligand (Ruderman *et al.*, 1990). Although *in vitro* assays of PtdIns 3-kinase activity in immunoprecipitates may not always reflect biological activity *in vivo*, the insulin-stimulated appearance of PtdIns 3-kinase activity in αPY immunoprecipitates has been shown to correlate with activation of the enzyme in intact cells (Ruderman *et al.*, 1990). PtdIns 3-kinase activity is also present in anti-insulin receptor immunoprecipitates at levels well above control antibodies matched for species and IgG class. Although specific, binding of the insulin receptor to the PtdIns 3-kinase is relatively weak compared with the PDGF receptor. Thus, the PDGF receptor will bind virtually all of the cellular PtdIns 3-kinase activity in epithelial cells (Kazlauskas and Cooper, 1990), whereas in CHO/IR cells the insulin receptor binds only 50% of the PtdIns 3-kinase activity detected with αPY antibody. Furthermore, activation of the PtdIns 3-kinase by the PDGF receptor correlates with the formation of a high-affinity complex between the two molecules (Kazlauskas and Cooper, 1990). In contrast, insulin receptor mutants with varying ability to activate the PtdIns 3-kinase show similar percentages of receptor-associated PtdIns 3-kinase activity. It is interesting to note that although the insulin receptor contains several tyrosine autophosphorylation sites, none of them are in the YMXM motif which is required for the association of the PtdIns 3-kinase with the PDGF receptor kinase insert region (Ullrich *et al.*, 1985). The insulin receptor does contain the sequence YTXM in its C terminus, but its removal in the $IR_{\Delta CT}$ mutant does not effect association of the receptor with the PtdIns 3-kinase. Thus, activation of the PtdIns 3-kinase by insulin may not depend on a direct association with the insulin receptor, or, like $pp60^{c\text{-}src}$, may be mediated by other proteins which do contain the YMXM motif.

In this context, it is interesting that activation of the PtdIns 3-kinase by insulin receptor mutants correlates more closely with the phosphorylation of pp185 and an 85-kDa band than with autophosphorylation of the insulin receptor. This may simply reflect the fact that these proteins are all substrates of the insulin receptor. Thus, the tyrosyl-phosphorylated 85–90-kDa band we see in insulin-stimulated CHO/IR cells may be the regulatory subunit of the PtdIns 3-kinase, although this has not yet been established. An 85-kDa PtdIns 3-kinase has been detected by middle T-blotting in αPY immunoprecipitates from insulin stimulated CHO/IR cells (Kapeler *et al.*, 1991). However, a more compelling explanation derives from our recent observation that immunoprecipitable PtdIns 3-kinase activity can be detected using antibodies to IRS-1, a recently cloned insulin receptor substrate which is a component of the 185-kDa insulin-stimulated tyrosyl phosphoprotein (Sun *et al.*, 1991). IRS-1 contains six potential tyrosyl phosphorylation sites containing the YMXM motif characteristic of PtdIns 3-kinase binding sites in other proteins (Cantley *et al.*, 1991). Thus the presence of PtdIns 3-kinase activity in αPY immunoprecipitates may in part result from a direct association between IRS-1 and the PtdIns 3-kinase. The significance of this association for insulin-stimulated activation of the PtdIns 3-kinase is not yet clear.

Although the cellular function of the PtdIns 3-kinase is not known, numerous studies on the biological activity of mutant PDGF and CSF-1 receptors and middle T antigens suggest that it is important in transformation and cellular responses to mitogens (Kaplan *et al.*, 1986; Coughlin *et al.*, 1989; Talmadge *et al.*, 1989; Coughlin *et al.*, 1989; Talmadge *et al.*, 1989; Shurtleff *et al.*, 1990; Reedijk *et al.*, 1990; Choudhury *et al.*, 1991). However, mutant CSF-1 receptors which display normal levels of receptor-associated PtdIns 3-kinase activity are defective for CSF-1-stimulated mitogenic responses, and mutant PDGF receptors defective for receptor-associated PtdIns 3-kinase activity retain some ligand-stimulated mitogenic responses (Roussel *et al.*, 1990; Heidaran *et al.*, 1991). Thus the relationship between necessity and sufficiency in the PtdIns 3-kinase signaling pathways remains elusive.

The panel of mutants in this study facilitates a comparison of PtdIns 3-kinase activation and insulin-stimulated biological effects (Fig. 7). The $IR_{\Delta CT}$, which lacks the two C-terminal autophosphorylation sites, stimulates the tyrosyl phosphorylation of pp185, the association of the PtdIns 3-kinase with αPY immunoprecipitates, and other biological effects normally. In contrast, the kinase-deficient mutant IR_{A1018} cannot undergo autophosphorylation and cannot transmit many insulin signals (Chou *et al.*, 1987), whereas a mutant containing a deletion in the juxtamembrane region ($IR_{\Delta 960}$) undergoes autophosphorylation normally, but phosphorylates the endogenous substrate pp185 weakly or not at all and is globally deficient in insulin-stimulated responses (Backer *et al.*, 1990; Backer *et al.*, 1991). These mutants support our previous hypothesis that autophosphorylation of the insulin receptor is necessary but not sufficient for signal transmission, as the juxtamembrane region of the insulin receptor is also essential for the tyrosyl phosphorylation or activation of endogenous substrates (White *et al.*, 1988; Backer *et al.* 1991). Specifically, autophosphorylation of the insulin receptor is not sufficient for recovery of the PtdIns 3-kinase in αPY immunoprecipitates, which also requires the ability to phosphorylate endogenous substrates, as detected by the phosphorylation of pp185 and the 85-kDa band.

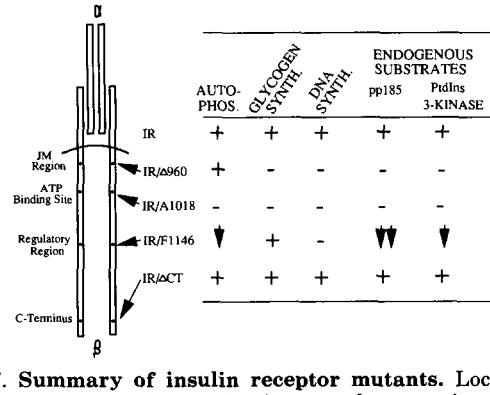

FIG. 7. **Summary of insulin receptor mutants.** Location of insulin receptor mutations in the juxtamembrane region ($IR_{\Delta 960}$), ATP binding site (IR_{A1018}), regulatory region (IR_{1146}), and C terminus ($IR_{\Delta CT}$) are shown in the *left panel.* Summary of biological activity with regard to autophosphorylation, metabolic activity (stimulation of glycogen synthesis), mitogenic activity (stimulation of thymidine incorporation into DNA), and endogenous substrate phosphorylation (pp185 and the PtdIns 3-kinase) are taken from: Chou *et al.*, 1987; Wilden *et al.*, 1990; Backer *et al.* 1990, 1991; McClain *et al.*, 1987; Myers *et al.*, 1991; and from Figs. 4 and 5.

We have reported previously (Wilden *et al.*, 1990) that cells expressing the regulatory region mutant IR_{F1146} were normally responsive to insulin for stimulation of glycogen synthesis but were defective in the tyrosyl phosphorylation of the endogenous substrate pp185 and the activation of thymidine incorporation into DNA. We now find that this mutant also shows a significant but incomplete reduction in the insulin stimulation of the PtdIns 3-kinase. Thus, a reduction in insulin stimulation of the PtdIns 3-kinase correlates with a deficiency in mitogenic responses to insulin. This relationship between cell growth and activation of the PtdIns 3-kinase is similar to that seen for the PDGF and CSF-1 receptors (Coughlin *et al.*, 1989; Shurtleff *et al.*, 1990; Reedijk *et al.*, 1990; Choudhury *et al.*, 1991) and provides further evidence for the role of the PtdIns 3-kinase in the cellular response to mitogenic stimuli.

In summary, we have shown that insulin stimulation of cells expressing the human insulin receptor leads to the rapid and sensitive stimulation of a PtdIns 3-kinase. The insulin-stimulated PtdIns 3-kinase is specifically immunoprecipitated by anti-receptor antibodies, and therefore appears to from a stable complex with the receptor. However, only 50% of the αPY-precipitable insulin-stimulated PtdIns 3-kinase activity is associated with the receptor. Simulation of the PtdIns 3-kinase by mutant insulin receptors show that: 1) full activation of the PtdIns 3-kinase, as detected in αPY or αIR immunoprecipitates, requires intact juxtamembrane and regulatory regions and ATP binding site, but does not require the C terminus region. 2) Mutants which are deficient in the the phosphorylation of the endogenous substrate pp185 and an 85 kDa band show reduced activation of the PtdIns 3-kinase. 3) Insulin-stimulated activation of the PtdIns 3-kinase may therefore be occur through a number of mechanisms, including direct phosphorylation by the insulin receptor or association with other proteins such as pp185 or one of its components, IRS-1. 4) A regulatory region mutant, IR_{F1146}, which does not undergo full kinase activation during insulin stimulation, cannot mediate insulin stimulation of PtdIns 3-kinase activity and is selectively defective in mitogenic signaling. Thus, the PtdIns 3-kinase may play an important role in the regulation of cell growth by insulin.

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