The Insulin and Insulin-like Growth Factor-I Receptor Substrate IRS-1 Associates with and Activates Phosphatidylinositol 3-Kinase in Vitro*

Sophie Giorgetti, Robert Ballotti, Aline Kowalski-Chauvel, Sophie Tartare, and Emmanuel Van Obberghen‡

From the Institut National de la Santé et de la Recherche Médicale Unité 145, Faculté de Médecine, Avenue de Valombrose, 06107 Nice Cédex 2, France

Phosphatidylinositol 3-kinase (PtdIns-3-kinase) is thought to participate in the transductional cascade used by several tyrosine kinase receptors including the insulin-like growth factor (IGF)-I receptor and the insulin receptor. The major insulin receptor cellular substrate IRS-1 (pp185) has been proposed as a possible link between the insulin receptor and PtdIns-3-kinase. In this study we show that both insulin and IGF-I treatment of murine fibroblasts transfected with insulin or IGF-I receptors increase PtdIns-3-kinase activity immunoprecipitated with an antibody directed against the 85-kDa subunit of PtdIns-3-kinase. Whereas only a small amount of PtdIns-3-kinase is found associated with the insulin and IGF-I receptor, a considerable PtdIns-3-kinase activity is immunoprecipitated by an antibody raised against IRS-1. Additionally, insulin and IGF-I stimulation of murine fibroblasts expressing insulin or IGF-I receptors induce tyrosine phosphorylation of IRS-1 and its association with PtdIns-3-kinase. Since IRS-1 seems to be the connection between PtdIns-3-kinase and insulin or IGF-I receptor, we used reconstitution experiments to characterize the implication of IRS-1 in the activation of PtdIns-3-kinase. We show that immunofinity-purified IRS-1 can be phosphorylated by ligand-stimulated insulin and IGF-I receptors and that this phosphorylation allows the association of IRS-1 with PtdIns-3-kinase. The interaction between PtdIns-3-kinase and IRS-1 phosphorylated by the insulin or the IGF-1 receptor results in the activation of PtdIns-3-kinase.

In conclusion, our results demonstrate that IRS-1 is a key component in the signal transduction pathway of PtdIns-3-kinase activation induced by insulin and IGF-I.

The binding of insulin to the α subunit of its transmembrane receptor induces activation of the tyrosine kinase activity contained in the receptor β subunit. This process results in multisite autophosphorylation of the insulin receptor β subunit and tyrosine phosphorylation of several endogenous substrates with M,, values ranging from 15,000 to 220,000 (Kahn, 1985; Gammeltoft and Van Obberghen, 1986; Rosen, 1987; Yarden and Ullrich, 1988). The first identified endogenous substrate of the insulin receptor was described in hepatoma cells by White et al. in 1985 (White et al., 1985, 1987). This substrate, called pp185, is a cytoplasmic protein that migrates in SDS-PAGE with an apparent molecular mass ranging from 165 to 200 kDa and which is rapidly tyrosine phosphorylated after treatment of cells with insulin or IGF-I (White et al., 1985; Izumi et al., 1987). Recently, Sun et al. (1991) cloned and sequenced a component of pp185 designated IRS-1 (insulin receptor substrate-1) (Rothenberg et al., 1991; Sun et al., 1991). The deduced protein sequence revealed the existence of 34 tyrosines, 14 included in consensus sequences YMXM or YXXM, which represent recognition motives for specific target proteins containing SH2 domains. These SH2 domains are noncatalytic regions of approximately 100 amino acids able to bind tyrosine-phosphorylated polypeptides. It is believed that SH2 domains may have a role in activation of signal transduction pathways by formation of multimeric protein complexes (Cantley et al., 1991; Koch et al., 1991).

The insulin receptor substrates, and particularly IRS-1, are thought to play a key role in transmission of the intracellular signals leading to the ultimate cellular responses evoked by the hormone. In addition to IRS-1, several proteins seem to be implicated in the hormone transductional cascade. These include (i) the small GTP-binding proteins of the Ras family which are activated by insulin (De Vries-Smits et al., 1992); (ii) the serine/threonine kinases of the extracellular signal-regulated kinase family (Boulton et al., 1991); and (iii) the phosphatidylinositol 3-kinase (PtdIns-3-kinase) (Endemann et al., 1990; Ruderman et al., 1990). The PtdIns-3-kinase belongs to the type I PtdIns kinases which phosphorylate the D-3 position of the inositol ring. The newly identified PtdIns-3-P, PtdIns-3,4-P₂, and PtdIns-3,4,5-P₃ may act as second messengers, but their roles in signal transmission remain to be defined. PtdIns-3-kinase is a heterodimer composed of an 85-kDa (p85) and a 110-kDa (p110) subunit (Escobedo et al., 1991b; Otsu et al., 1991; Skolnik et al., 1991). Cloning and sequencing of the 85-kDa subunit revealed that there are two different forms of this subunit, p85A and p85B. Both p85A and p85B...
isoforms contain two SH2 domains and appear to play a regulatory role in the enzymatic activity contained in the p110 subunit. Recently, this subunit has been cloned and sequenced, and it has been shown to possess enzymatic activity in the presence of the p85 subunit (Hiles et al., 1992). Further, it displays significant sequence homology with the Saccharomyces cerevisiae protein Vsp34p, which has been implicated in targeting of proteins and in vesicular transport.

The PtdIns-3-kinase activity was found to associate with numerous tyrosine kinases such as pp60csrc (Fukui and Hanafusa, 1989), complex pp60src-middle T (Courtenidge and Heber, 1987; Whitman et al., 1985), and oncogene products and growth factor receptor tyrosine kinases (Cantley et al., 1991). A direct association between p85 and the platelet-derived growth factor receptor, and between p85 and the colony-stimulating factor-1 receptor has been shown (Kazlauskas and Cooper, 1990; Escobedo et al., 1991a; Hu et al., 1992; McGade et al., 1992). In the case of the insulin receptor and the IGF-I receptor, it has been demonstrated that ligand stimulation of fibroblasts overexpressing these receptors induces an augmentation in the PtdIns-3-kinase activity in antiphosphotyrosine immunoprecipitates, indicating that the enzyme is tyrosine phosphorylated and/or associated with a tyrosine-phosphorylated protein (Endemann et al., 1990; Ruderman et al., 1992; Yamamoto et al., 1992). For the insulin receptor, this phosphorylated protein could be IRS-1, since PtdIns-3-kinase activity was described to be tightly associated with IRS-1 in immunoprecipitates both in fibroblasts (Sun et al., 1991) and in adipocytes exposed to insulin (Giorgetti et al., 1992). Finally, using an antibody to the p85 subunit of PtdIns-3-kinase, we have been able to demonstrate that in adipocytes insulin activates this enzyme (Giorgetti et al., 1992). To the best of our knowledge concerning IGF-I, the possible implication of IRS-1 in the activation of PtdIns-3-kinase had not been explored. To gain insight into the mechanism of PtdIns-3-kinase activation by insulin or IGF-I and into the respective contributions of the different players, the insulin receptor, the IGF-I receptor, and IRS-1, we performed in vitro reconstitution experiments using immunopurified proteins.

We show that in intact cells IGF-I as well as insulin stimulate the PtdIns-3-kinase activity and its association with IRS-1. Furthermore, we demonstrate that IRS-1 is phosphorylated in vitro by the insulin receptor and by the IGF-I receptor. The tyrosine-phosphorylated IRS-1 is able to associate with the p85 subunit of PtdIns-3-kinase, whereas this association occurs poorly with the insulin or IGF-I receptor alone. Moreover, the association of tyrosine-phosphorylated IRS-1 with p85 appears as an event that is sufficient for the activation of the enzymatic activity of PtdIns-3-kinase.

Taken together our data demonstrate that (i) insulin and IGF-I use a similar pathway to activate the PtdIns-3-kinase, and (ii) transmission of the signal from insulin or IGF-I receptors to PtdIns-3-kinase necessitates the participation of IRS-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—PtdIns from bovine liver, Nonidet P-40, Triton X-100, protein A-Sepharose CL-4B, and aprotinin were purchased from Sigma. Bovine serum albumin was purchased from Intergen, and [γ-32P]ATP (7,000 Ci/mmol) was from ICN. Phenylmethylsulfonyl fluoride was from Serva, Heidelberg, Germany; and porcine insulin was from Novo-Nordisk, Copenhagen, Denmark.

**Antibodies**—Antibodies to the p85 subunit of PtdIns-3-kinase (anti-p85) were kindly provided to us by Professor Joseph Schlessinger and Dr. Ben Margolis (NYU, New York). Antibody 51 is directed against a peptide corresponding to the sequence 513-531 of the p85 subunit of PtdIns-3-kinase (Hu et al., 1992). Antibody to IRS-1 was obtained by injection of a rabbit with the peptide Pep88 coupled to keyhole limpet hemocyanin with EDAC. The synthetic peptide Pep88 corresponds to the amino acid sequence 486-504 derived from the cDNA of IRS-1 (Sun et al., 1991). The monoclonal antibody B6 is directed against the α-subunit of the insulin receptor (Gautier et al., 1986). The monoclonal antibody 2431 against the IGF-I receptor was provided to us by Professor K. Scholle (University of London). This antibody is directed against the deduced epitope 283-440 or 586-908 of the IGF-I receptor (Soos et al., 1992).

**Cell Culture**—NIH3T3 cells are mouse embryo fibroblasts (NIH3T3) transfected with an expression plasmid coding for the human insulin receptor; they were a gift from Dr. J. Whittaker (Stony Brook, NY). NIHGF-IR are NIH3T3 cells transfected with an expression vector pCDNAneo (Invitrogen, San Diego, CA) carrying the human IGF-I receptor cDNA and the neomycin resistance gene. The IGF-I receptor cDNA was obtained from Dr. Pierre De Meyts (Copenhagen, Denmark). The cells were grown in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% fetal calf serum.

**Immunoprecipitation**—Confluent cells growing in 145-mm culture dishes were stimulated or not with insulin (10⁻⁷ M) or IGF-I (10⁻⁷ M) for the indicated times after stimulation, the cells were cooled to 4 °C and washed with re-cold phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 6 mM NaH₂PO₄, 1 mM KH₂PO₄, pH 7.4) and homogenized with lysis buffer (50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM Na₂PO₄, 100 mM NaF, 2 mM vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 100 IU/ml Trasylol, 1% Nonidet P-40, pH 7.4, 1 ml/145-mm dish) for 15 min at 4 °C. The solubilized proteins were immunoprecipitated with the different antibodies preadsorbed on protein A- or protein G-Sepharose. Phosphatidylinositol 3-Kinase Assay—The PtdIns-3-kinase activity was measured after immunoprecipitation with the indicated antibodies for 90 min at 4 °C as described previously (Giorgetti et al., 1992). The immunoprecipitates were washed twice with each of the following three following buffers: (i) phosphate-buffered saline (pH 7.4) containing 1% Nonidet P-40; (ii) 100 mM Tris, 0.5 M LiCl, pH 7.4; and (iii) 10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4 (Whitman et al., 1985; Endemann et al., 1990). The pellets were resuspended in 30 µl of 20 mM Hepes, 0.4 mM EGTA, 0.4 mM Na₂HPO₄ and the kinase reaction was started by addition of PtdIns (0.2 µM), 10 mM MgCl₂ and 50 µM [γ-32P]ATP (10 Ci/mmol). After 15 min, the reaction was stopped by the addition of 15 µl of 4 N HCl and the phosphoinositides were extracted with 130 µl of chloroform:methanol (1:2). The phospholipids were analyzed by thin layer chromatography and autoradiography (Whittman et al., 1985, 1987). The positions of PtdIns-P and PtdIns-2P were determined by comparison with the migration standards (Sigma). It should be noted that the appearance of a PtdIns-P₂ spot is dependent on the amount of PtdIns-F in the mixture used as substrate.

**Reconstitution Experiments**—Insulin or IGF-I receptors were obtained by the two following methods: (i) immunoprecipitation from extracts of cells exposed or not to insulin or to IGF-I; (ii) chromatography on WGA-Sepharose of cell extracts followed by in vitro stimulation with their respective ligands for 45 min at 15 °C. IRS-1 was obtained after immunoprecipitation from unstimulated cells. Receptors were incubated with or without IRS-1 and phosphorylated in the presence of 8 nM, MgCl₂, 4 mM MnCl₂, and 15 µM ATP for 15 min at room temperature. The pellets were washed and incubated for 5 min at room temperature with a lysate from unstimulated cells or with an anti-p85 (antibody 51) immunoprecipitate. The pellets were washed and assayed for the PtdIns-3-kinase activity as described above.

**Metabolic Labeling of Cells with [³⁵S]Methionine and [³⁵S]Cysteine**—Confluent cells were incubated overnight in methionine and cysteine-free Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, [³⁵S]methionine, and [³⁵S]cysteine (Trans-[³⁵S]label, ICN, 1 mCi/100-mm dish). After stimulation by insulin or IGF-I, the cells were washed with phosphate-buffered saline, solubilized, and immunoprecipitated with the indicated antibodies. After immunoprecipitation, the pellets were washed with HNT (30 mM Hepes, 30 mM NaCl, 100 mM EDTA, pH 7.4) and lysis buffer containing 0.5 mM NaCl, 0.2% SDS, 0.1% Triton X-100. The proteins were eluted with Laemmli buffer containing 3% SDS and 5% β-mercaptoethanol (Laemmli, 1970). The samples were analyzed by SDS-PAGE followed by autoradiography.

**Blotting Analysis**—After immunoprecipitation with the indicated antibodies and separation of the proteins by SDS-PAGE, proteins were transferred to an Immobilon membrane (Immobilon polyvinylidene difluoride, Millipore). The membrane was saturated...
and blotted with the indicated antibodies. Finally, proteins were visualized by using \(^{125}\)I-protein A followed by autoradiography.

**RESULTS**

**Insulin- and IGF-I-stimulated PtdIns-3-kinase Activity in Intact Cells**—First, we investigated the capacity of both insulin and IGF-I to stimulate the PtdIns-3-kinase activity in mouse fibroblasts expressing human insulin receptors (NHIR) or IGF-I receptors (NHIGF-IR). Cells were stimulated respectively with insulin or IGF-I, and the activation of PtdIns-3-kinase was measured after immunoprecipitation with antibodies to one of the following proteins: insulin receptor, IGF-I receptor, IRS-1, or the p85 subunit of PtdIns-3-kinase (Fig. 1).

Immunoprecipitation with an antibody to p85 showed that in insulin- and IGF-I-treated cells PtdIns-3-kinase activity was dramatically stimulated (Fig. 1, lanes E, F, K, and L). An important amount of this PtdIns-3-kinase activity was found to be associated with the immunoprecipitated IRS-1 (lanes C, D, I, and J). In contrast, insulin and IGF-I induced a small increase in PtdIns-3-kinase activity recovered after immunoprecipitation of the corresponding receptors (lanes A, B, G, and H).

These results indicate that insulin and IGF-I promote the activation of PtdIns-3-kinase and that PtdIns-3-kinase activity is tightly associated to IRS-1 but interacts only poorly with insulin or IGF-I receptors.

**In Vivo Association of IRS-1 and PtdIns-3-kinase**—To characterize further the interactions between the receptor for insulin and IGF-I, their endogenous substrate IRS-1, and PtdIns-3-kinase, we metabolically labeled NHIR cells with \(^{35}\)S)methionine and \(^{35}\)S)cysteine. In anti-IRS-1 immunoprecipitates, we observed a labeled protein at 170 kDa corresponding to IRS-1 (Fig. 2, panel A, lane A). Insulin treatment of the cells induced a shift of IRS-1 to a higher molecular mass (185 kDa) (lane B). This slower mobility was probably caused by a change in the phosphorylation state of the protein. Moreover, under this condition, two proteins with Mr values of 85,000 and 110,000, very likely corresponding to the two PtdIns-3-kinase subunits, were immunoprecipitated together with IRS-1 (lane B).

In a second approach, the interactions between IRS-1 and PtdIns-3-kinase were analyzed by Western blotting experiments. NHIR or NHIGF-IR cells were incubated or not with insulin or IGF-I, and the cell extracts were subjected to immunoprecipitation with antibodies to p85 and to IRS-1. The 85-kDa PtdIns-3-kinase subunit was revealed by Western blotting with an antibody to p85 (Fig. 2, panel B). We found that treatment of cells with insulin (lanes A and B) or IGF-I (not shown) did not modify the ability of our antibody to recognize the p85 subunit of PtdIns-3-kinase. In addition, we observed that insulin (lanes C and D) or IGF-I (lanes E and F) increased the amount of PtdIns-3-kinase detected by blotting with anti-p85 in anti-IRS-1 precipitates. Taken together these data demonstrate that insulin and IGF-I stimulate the association between PtdIns-3-kinase and IRS-1.

Next, we were interested to see whether PtdIns-3-kinase becomes tyrosine phosphorylated in intact cells. NHIR or NHIGF-IR cells were stimulated by insulin (10\(^{-7}\) M) or IGF-I (10\(^{-7}\) M) and thereafter solubilized. The cell extracts were subjected to immunoprecipitation with antibodies to insulin receptor, IGF-I receptor, p85, and IRS-1, and the tyrosine-phosphorylated proteins were revealed by Western blotting with an antibody to phosphotyrosine (Fig. 3). After immunoprecipitation with anti-IRS-1 antibody, we found that insulin and IGF-I stimulated the tyrosine phosphorylation of IRS-1 (Fig. 3, lanes A, B, G, and H). In both anti-insulin receptor or anti-IGF-I receptor precipitates, the only tyrosine-phosphorylated proteins found correspond to the receptors \(\beta\) subunits (Fig. 3, lanes C, D, I, and J). In anti-p85 immunoprecipitates, we observed a strong labeling of a 185-kDa protein corresponding to IRS-1 but no tyrosine phosphorylation of the 85-kDa subunit of the PtdIns-3-kinase (lanes E, F, K, and L). The absence of tyrosine-phosphorylated insulin or IGF-I receptor \(\beta\) subunits in anti-IRS-1 and anti-p85 pellets suggests that there is no or little association between the receptors and the IRS-1-PtdIns-3-kinase complex. This was confirmed by the absence of tyrosine phosphorylated IRS-1 in an antireceptor precipitates.

**In Vitro Reconstitution of PtdIns-3-kinase Activation**—To approach the mechanism by which insulin and IGF-I activate PtdIns-3-kinase, reconstitution experiments were performed reproducing in vitro the activation of PtdIns-3-kinase by insulin or IGF-I receptors.

Insulin or IGF-I receptors were immunoprecipitated from unstimulated cells or cells stimulated with insulin or IGF-I for 10 min at 37°C. First, as shown previously in Fig. 1, we found that a small amount of PtdIns-3-kinase activity was directly associated to the insulin receptor and the IGF-I receptor (Fig. 4, lanes A, B, G, and H). This association was specific, since with a nonimmune serum no PtdIns-3-kinase activity could be detected (data not shown). When immunoprecipitated insulin or IGF-I receptors were first phosphorylated and then incubated in the presence of a cell lysate from unstimulated cells as the source of exogenous PtdIns-3-kinase (lanes C, D, I, and J), the receptor autophosphorylation induced a moderate increase in the PtdIns-3-kinase associated with the receptors. Finally, immunoprecipitated receptors were incubated with an anti-IRS-1 immunoprecipitate from unstimulated cells and phosphorylated before the addition of the lysate as the source of exogenous PtdIns-3-kinase. In this
Interaction of Phosphatidylinositol 3-Kinase with IRS-1

A A B B antibodies to immunoprecipitation with -I, P85 IRS1

FIG.

2. Association of PtdIns-3-kinase and IRS-1 in intact cells. Panel A, confluent NHIR cell dishes were labeled overnight with [35S]methionine and [35S]cysteine. Cells were stimulated (lane B) or not (lane A) with insulin (10^{-7} M) for 10 min at 37°C before solubilization. The homogenates were immunoprecipitated with an antibody to IRS-1 (serum dilution 1/50) for 90 min at 4°C. After washes, the proteins were analyzed by SDS-PAGE followed by autoradiography.

Panel B, NHIR or NHGF-IR cells were stimulated or not with insulin (10^{-7} M) or IGF-I (10^{-9} M) for 10 min before homogenization. Lysates were immunoprecipitated with antibodies to p85 (lanes A and B) and to IRS-1 (lanes C-F) as indicated. Proteins were revealed by Western blotting with an antibody to p85 as described under "Experimental Procedures."

These data suggest that phosphorylation of IRS-1 by insulin or IGF-I receptors is the signal that allows the association of IRS-1 with PtdIns-3-kinase. However, as receptors are present in the pellets it could be argued that they are involved in this association process. To rule this hypothesis out and to confirm that phosphorylation of IRS-1 is required for the association with PtdIns-3-kinase, we have performed the following experiments. First, we examined the ability of the insulin and the IGF-I receptors to phosphorylate IRS-1 in vitro. The insulin and IGF-I receptors were partially purified from NHIR and NHGF-IR cells, respectively, by wheat germ agglutinin chromatography. Purified receptors were stimulated with insulin or with IGF-I and incubated with IRS-1 immunoprecipitated from unstimulated cells. The samples were phosphorylated in the presence of [γ-32P]ATP for 30 min at room temperature and washed to remove the receptors and to retain only IRS-1 bound to protein A. As shown in Fig. 5, when the reaction was performed in the presence of...
unstimulated insulin or IGF-I receptors, we observed the phosphorylation of a 180-kDa protein corresponding to IRS-1 (lanes A and C). Further, this phosphorylation was markedly increased by the addition of activated receptor (lanes B and D), indicating that in vitro insulin and IGF-I receptors were able to phosphorylate IRS-1.

In the following series of experiments, we wished to exclude a possible participation of the receptors in the IRS-1-PtdIns-3-kinase association and to analyze in more detail the effect of this association on PtdIns-3-kinase activation. WGA-purified insulin or IGF-I receptors were stimulated or not with their respective ligands and incubated with anti-IRS-1 immunoprecipitates from unstimulated cells. In vitro phosphorylation was performed as shown in Fig. 5, and insulin or IGF-I receptors were removed before the incubation of IRS-1 with a cell lysate as the source of exogenous PtdIns-3-kinase. We found that phosphorylation of IRS-1 by activated receptors increased markedly its ability to bind PtdIns-3-kinase (Fig. 6, lanes A-D), indicating that IRS-1 alone interacts with PtdIns-3-kinase.

Finally, we looked at the effect of the association between IRS-1 and PtdIns-3-kinase on the activity of the enzyme. Briefly, immunoprecipitated IRS-1 was phosphorylated by insulin and IGF-I receptors as described previously. After the phosphorylation reaction, insulin or IGF-I receptors were removed, and IRS-1 was incubated with PtdIns-3-kinase immunoprecipitated from unstimulated cells. As illustrated in Fig. 6 (lanes E–H), we found that IRS-1 phosphorylated by activated insulin receptor or by activated IGF-I receptor increased the PtdIns-3-kinase activity. In conclusion, we have shown here that the insulin- or IGF-I-induced-phosphorylation of IRS-1 promotes its interaction with PtdIns-3-kinase. This association appears to be sufficient to activate the enzyme.

DISCUSSION

In the first part of this work we studied the effects of insulin and IGF-I on PtdIns-3-kinase in intact cells. Data from several laboratories including our own indicate that insulin activates PtdIns-3-kinase and induces its association with IRS-1 (Endemann et al., 1990; Giorgetti et al., 1992; Kelly et al., 1992; Ruderman et al., 1990; Sun et al., 1991). Although it has been shown previously that IGF-I treatment of mouse fibroblasts overexpressing IGF-I receptors (LISN C4) increases PtdIns-3-kinase activity in anti-phosphotyrosine precipitates, no evidence has been reported indicating that IGF-I activates the PtdIns-3-kinase and induces its association with IRS-1 (Yamamoto et al., 1992). Here we show that stimulation by insulin and IGF-I greatly augments the PtdIns-3-kinase activity in anti-phosphotyrosine precipitates and that a large amount of this activity is found to be associated with anti-IRS-1 pellets. Western blotting experiments with anti-p85 show that insulin and IGF-I enhance the amount of PtdIns-3-kinase in anti-IRS-1 precipitates. Taken together, our findings indicate that IGF-I, similarly to insulin, stimulates the enzymatic activity of the PtdIns-3-kinase and its association with IRS-1.

Next we showed that insulin or IGF-I treatment of NIH3T3 cells expressing insulin or IGF-I receptors induces tyrosine phosphorylation of IRS-1 and that this tyrosine-phosphorylated IRS-1 is associated with PtdIns-3-kinase. In contrast, we did not detect the association of insulin or IGF-I receptor either with IRS-1 or with PtdIns-3-kinase. In fact, the small amount of PtdIns-3-kinase activity immunoprecipitated with antibodies to insulin receptor and to IGF-1 receptor in NIH3T3 cells did not permit the detection of this association by Western blotting. The direct or indirect association of PtdIns-3-kinase with receptors implies translocation of the enzyme to the cell membrane. This is in agreement with previous studies showing that insulin treatment induces a recruitment of PtdIns-3-kinase from the cytosol to the plasma membrane (Giorgetti et al., 1992). In addition, a recent study by Backer et al. (1992a) in Chinese hamster ovary cells transfected with the insulin receptor cDNA suggests the existence of a ternary complex involving insulin receptor-IRS-1-PtdIns-3-kinase. It should be noted that at least in human

FIG. 4. In vitro association of PtdIns-3-kinase and IRS-1 phosphorylated by insulin and IGF-I receptors. NIH cells (left panel) or NIHIGF-IR cells (right panel) were incubated in the absence or in the presence of insulin (10^{-7} M) or IGF-I (10^{-7} M) for 10 min at 37°C. Cells were solubilized and homogenates were immunoprecipitated with an antibody to insulin receptor (ascites dilution 1/150) or to IGF-I receptor (ascites dilution 1/150). Lanes A, B, G, and H, measurement of PtdIns-3-kinase activity. Lanes C, D, I, and J, immunoprecipitated receptors were phosphorylated for 15 min at room temperature with 8 mM MgCl_{2}, 4 mM MnCl_{2}, and 15 μM ATP (final concentrations) and incubated with a cytosol (v/v) from unstimulated cells for 5 min. The pellets were washed prior to measurement of the PtdIns-3-kinase activity. Lanes E, F, K, and L, immunoprecipitated receptors were incubated with immunoprecipitated IRS-1, phosphorylated for 15 min at room temperature, and incubated for 5 min with a cytosol (v/v) from unstimulated cells. Finally, the pellets were washed prior to measurement of the PtdIns-3-kinase activity. PIP, PtdIns-P; PIP₂, PtdIns-P₂.
kidney cells of the 293 line and in Chinese hamster ovary cells (Backer et al., 1992a) but not in NIH3T3 cells (this study). insulin or IGF-I receptors are tightly associated to IRS-1 and PtdIns-3-kinase. These observations might indicate that the association of PtdIns-3-kinase with these receptors is dependent of a specific cellular component that is absent in NIH3T3 cells but exists in other cell lines such as Chinese hamster ovary and 293 cells. Interestingly, we did not detect tyrosine phosphorylation of p85 and p110 after immunoprecipitation with anti-p85.

Taken together, the results presented above indicate that similarly to the observations made with the insulin receptor, (i) p85 is not a direct substrate for the IGF-I receptor tyrosine kinase, and (ii) the IGF-I receptor interacts poorly with PtdIns-3-kinase. These results differ from those observed with other tyrosine kinase receptors such as the platelet-derived growth factor receptor. Indeed, it has been shown that after ligand stimulation, the platelet-derived growth factor receptor is able to phosphorylate p85 on tyrosine residues (Kaplan et al., 1987) and to associate directly with the SH2 domains of PtdIns-3-kinase through tyrosyl phosphorylation of the sequence motif Y751MXM (Kazlauskas and Cooper, 1990). A mutant of the platelet-derived growth factor receptor lacking this phosphorylation site does not bind nor activate PtdIns-3-kinase (Kazlauskas and Cooper, 1990).

Since IRS-1 appears to function as the physical link between PtdIns-3-kinase and the receptor for IGF-I and insulin, we performed in vitro reconstitution experiments to characterize further the role of IRS-1 in PtdIns-3-kinase activation. Having shown that IRS-1 can be phosphorylated in vitro directly by activated insulin or IGF-I receptors, we investigated the role of phosphorylated IRS-1 in the association and activation of PtdIns-3-kinase. Using in vitro reconstitution experiments we found that incubation of autophosphorylated insulin or IGF-I receptor with an unstimulated cell lysate as a source of PtdIns-3-kinase increases the amount of associated PtdIns-3-kinase compared with the activity found associated in intact cells. The direct interaction between PtdIns-3-kinase and insulin or IGF-I receptor could be because of the presence of the YXXX sequence in the β subunit of both receptors. This sequence is indeed quite close to the canonical motif YMXM, which participates in interactions with the SH2 domains contained in several proteins and in particular with the SH2 domains of PtdIns-3-kinase (Cantley et al., 1991; Koch et al., 1991). This sequence does not appear to be involved in the binding of PtdIns-3-kinase in intact cells, since removal of the carboxyl-terminal region of the insulin receptor containing this sequence does not affect the activation of PtdIns-3-kinase by the mutated receptor (Backer et al., 1992b).

Furthermore, the presence of phosphorylated IRS-1 in our reconstitution experiment results in a marked increase in PtdIns-3-kinase activity. These data indicate that in vitro phosphorylated IRS-1 is able to interact directly with PtdIns-3-kinase. Indeed, when the receptor was removed after IRS-1 phosphorylation, a strong association of PtdIns-3-kinase with IRS-1 is conserved. These observations are in agreement with a recent study of Lavan et al. (1992) showing that the tyrosine-phosphorylated form of pp160, the mouse homologue of IRS-
1, was associated in a 10 S complex with PtdIns-3-kinase in a fraction devoid of receptor (Lavan et al., 1992).

In all of our reconstitution experiments an unstimulated cell lysate was used as source of PtdIns-3-kinase, and we show that the increase in PtdIns-3-kinase activity in anti-IRS-1 precipitates was because of a better association of PtdIns-3-kinase with phosphorylated IRS-1. We also wanted to examine the effect of IRS-1 phosphorylation on PtdIns-3-kinase activation. We found that tyrosine-phosphorylated IRS-1 was able to increase the activity contained in anti-p85 immunoprecipitates, whereas receptors alone incubated with anti-p85 immunoprecipitates do not increase the PtdIns-3-kinase activity (data not shown). These observations are in agreement with the work of Backer et al. (1992a), illustrating that IRS-1 phosphorylated by insulin receptor can activate in vitro PtdIns-3-kinase. Our result indicates that, after its tyrosine phosphorylation by insulin or IGF-I receptor are similar, and they emphasize the crucial role of IRS-1 in the mechanism of PtdIns-3-kinase activation by both insulin and IGF-I receptors.

In conclusion, our results demonstrate that PtdIns-3-kinase associates in vitro with phosphorylated IRS-1 and with insulin and IGF-I receptors. However, the association of PtdIns-3-kinase with receptors was small compared with the association between PtdIns-3-kinase and IRS-1. Finally, the interaction between PtdIns-3-kinase and tyrosine phosphorylated IRS-1 appears to be sufficient to activate the PtdIns-3-kinase. The interaction of IRS-1 with PtdIns-3-kinase places this enzyme very early in the transductional cascade. In this context the identification of the elements immediately downstream of PtdIns-3-kinase becomes an urgent issue for a better understanding of the insulin and IGF-1 signaling pathway.

Acknowledgments—We thank Professor J. Schlessinger and Dr. B. Margolis for antibodies to p85 (antibody 51); Dr. M. White, for the peptide to IRS-1 (Pep80); Dr. P. DeMeyts, for the IGF-I cDNA; and Dr. Siddle, for antibodies to IGF-I receptor. We thank Drs. Y. Le Marchand-Brustel, J. F. Tanti, E. Schenker, E. Van Obberghen-Schilling, and F. Penaldi for critical reading of the manuscript; J. Duch, for secretarial assistance; and C. Minghelli and G. Viscioni, for illustration work.

REFERENCES

Backer, J. M., Myers, M. G., Shoelson, S. E., Chin, D. J., Sun, X. J., Mirsalipesi, M., Hu, P., Margolis, B., Skolnick, E. Y., Schlessinger, J., and White, M. (1992a) EMBO J. 11, 3469-3479

Backer, J. M., Schroeder, G. G., Kahn, C. R., Myers, M. G., Jr., Wilden, P. A., Cahill, D. A., and White, M. F. (1992b) J. Biol. Chem. 267, 1367-1374

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewski, E., Ello, K., Jones, S. D., DeFrisco, R. A., Panayotatos, N., Cobb, M. H., and Yancopoulos, G. D. (1991) Cell 65, 663-670

Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kawecki, K., and Sollott, S. (1991) Cell 64, 261-262

Courteaud, S. A., and Heber, A. (1987) Cell 50, 1031-1037

De Vries-Strats, A. M. M., Burgers, B. M. T., Levers, S. J., Marshall, C. J., and Bissell, J. J. L. (1992) Nature 357, 602-607

Endemann, G., Yonezawa, K., and Roth, R. A. (1990) J. Biol. Chem. 265, 398-404

Eccobedo, J. A., Kaplan, D. R., Kavanagh, W. M., Turck, C. W., and Williams, T. L. (1991a) Mol. Cell. Biol. 11, 1125-1132

Eccobedo, J. A., Navanka-Koulas, S., Kavanagh, W. M., Milway, D., Fried, V. A., and Williams, L. T. (1991b) Cell 65, 75-82

Fukui, Y., and Hanafusa, H. (1989) Mol. Cell. Biol. 9, 1651-1658

Gauselof, S., and Van Obberghen, E. (1986) Biochem. J. 235, 1-11

Gautier, N., Alengrin, F., Ponzio, G., Rossi, B., and Dolakis-K投标gi, J. (1986) Ann. Endocrinol. 47, 21-26

Giorgetti, S., Ballotti, R., Kowalski-Chauvel, A., Corzont, M., and Van Obberghen, E. (1992) Eur. J. Biochem. 207, 599-606

Hiles, I. D., Otou, M., Volinia, S., Fry, M. J., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N. F., Hsu, J. J., Courteaud, S. A., Parker, P. J., and Waterfield, M. D. (1992) Cell 70, 419-429

Hu, P., Margolis, B., Skolnick, E. Y., Lammers, R., Ulrich, J., and Schlessinger, J. (1992) Mol. Cell. Biol. 12, 981-990

Izumi, T., White, M. F., Kadokawa, T., Takaku, F., Akamaiya, Y., and Kasuga, M. (1987) J. Biol. Chem. 262, 1292-1297

Kahn, C. R. (1986) Annu. Rev. Med. 37, 429-451

Kaplan, D. N., Whitman, M., Schaffhausen, B., Pallas, D. C., White, M., Cantley, L. C., and Roberts, P. Y. (1987) Cell 56, 1021-1029

Kaszas, A., and Cooper, J. A. (1990) EMBO J. 9, 3279-3286

Koehler, P., Ruderman, N. B., and Chen, K. S. (1992) J. Biol. Chem. 267, 3432-3438

Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991) Science 252, 668-674

Laemmli, U. K. (1970) Nature 227, 680-685

Lavan, B. E., Kohne, R. M., Glaser, C. W., Anderson, D. Reedijk, M., Pawson, T., and Lienard, G. H. (1992) J. Biol. Chem. 267, 11531-11536

McGlade, C. J., Ellis, C., Reedijk, M., Anderson, D., Bhaman, G., Read, A. D., Panayotou, G., Gerd, P., Bernstein, A., Kaslanska, A., Waterfield, M. D., and Pawson, T. (1992) Mol. Cell. Biol. 12, 991-997

Otou, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thiemann, A., Dhand, R., Totty, N. Smith, A. L., Morgan, S. J., Courteaud, S. A., Parker, P. J., and Waterfield, M. D. (1991) Cell 65, 91-104

Rosen, O. M. (1987) Science 237, 1452-1458

Rothenberg, P. L., Lane, W. S., Karak, A., Backer, J. White, M., and Kahn, C. R. (1991) J. Biol. Chem. 266, 8300-8311

Ruderman, N. B., Kapeller, R., White, M. F., and Cantley, L. C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1411-1415

Sklar, S. Y., Margolis, B., Mohyeldin, M., Lowenstein, E., Fischer, R., Dreppa, A., Ulrich, A., and Schlessinger, J. (1991) Cell 65, 83-90

Sosa, M. A., Field, C. E., Lammers, R., Ulrich, A., Zhang, B., Roth, R. A., Andersen, A. S., Kjeldsen, T., and Siddle, K. (1993) Cell 70, 12955-12963

Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) Nature 352, 73-77

White, M. F., Maron, R., and Kahn, C. R. (1985) Nature 318, 183-186

White, M. F., Stegmann, E. W., Dull, T. J., Ulrich, A., and Kahn, C. R. (1987) J. Biol. Chem. 262, 9769-9777

Whitman, M., Kaplan, D. R., Schaffhausen, B., Cantley, L. C., and Roberts, T. M. (1985) Nature 315, 239-242

Whitman, M., Kaplan, D., Roberts, T., and Cantley, L. C. (1987) Biochem. J. 247, 163-174

Whitman, M., Downes, C. P., Keeler, M., Keller, T., and Cantley, L. C. (1988) Nature 332, 644-646

Yamamoto, K., Lapetina, E. G., and Mowham, C. P. (1992) Endocrinology 130, 1490-1498

Yarden, Y., and Ulrich, A. (1988) Annu. Rev. Biochem. 57, 443-478